



PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification<sup>4</sup> :</b> <b>C12Q 1/68, C07H 15/12</b> <b>G01N 33/48</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 90/01069</b> <b>(43) International Publication Date:</b> 8 February 1990 (08.02.90)
<b>(21) International Application Number:</b> PCT/US89/03125 <b>(22) International Filing Date:</b> 19 July 1989 (19.07.89)  <b>(30) Priority data:</b> 221,750                      20 July 1988 (20.07.88)                      US  <b>(71) Applicant:</b> SEGEV DIAGNOSTICS, INC. [US/US]; Greenfeld, 1115 52nd Street, Brooklyn, NY 11219 (US).  <b>(72) Inventor:</b> SEGEV, David ; Moshav Bnei Mrem 40, 79 840 (IL).  <b>(74) Agents:</b> LIEBERSTEIN, Stanley, H. et al.; Ostrolenk, Fa- ber, Gerb & Soffen, 1180 Avenue of the Americas, New York, NY 10036 (US).		<b>(81) Designated States:</b> AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), (European patent), JP, LU (European patent), NL (European patent), SE (European patent).  <b>Published</b> <i>With international search report.</i>

**(54) Title:** PROCESS FOR AMPLIFYING AND DETECTING NUCLEIC ACID SEQUENCES**(57) Abstract**

This invention relates to a process for amplifying and detecting any desired specific nucleic acid sequence that exists in nucleic acid or mixture thereof. The process comprises treating single strand RNA or separated complementary strands of DNA target with a molar excess of oligonucleotide complement pairs in which these oligonucleotide complement pairs have sequence complementary to the target, under hybridizing conditions. In one embodiment, the oligonucleotide complement pairs may have a gap of one or more bases which may be repaired (filled) by enzymes. The oligonucleotide complement pairs are joined together forming joined, oligonucleotide product. The target/joint product hybrid nucleic acids are then denatured to single strands again at which point both the target and the joined products can form hybrids with new oligonucleotide complement pairs. The steps of the reaction may be carried out stepwise or simultaneously and can be repeated as often as desired.

BEST AVAILABLE COPY

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MR	Mauritania
BE	Belgium	GA	Gabon	MW	Malawi
BF	Burkina Faso	GB	United Kingdom	NL	Netherlands
BG	Bulgaria	HU	Hungary	NO	Norway
BJ	Benin	IT	Italy	RO	Romania
BR	Brazil	JP	Japan	SD	Sudan
CA	Canada	KP	Democratic People's Republic of Korea	SE	Sweden
CF	Central African Republic	KR	Republic of Korea	SN	Senegal
CG	Congo	LI	Liechtenstein	SU	Soviet Union
CH	Switzerland	LK	Sri Lanka	TD	Chad
CM	Cameroon	LU	Luxembourg	TG	Togo
CV	Cape Verde			US	United States of America

- 1 -

PROCESS FOR AMPLIFYING AND  
DETECTING NUCLEIC ACID SEQUENCES

FIELD OF THE INVENTION

5       The present invention is related to a process  
for amplifying and detecting existing nucleic acid  
sequences if they are present in a test sample. More  
specifically, it is related to a process for producing  
any particular nucleic acid sequence from a given  
sequence of DNA or RNA in amounts which are large, when  
10       compared to the amount initially present. The DNA or  
RNA may be single or double-stranded, and may be a  
relatively pure species or a component of a mixture of  
nucleic acids. The process of the invention utilizes a  
repetitive reaction to accomplish the amplification of  
15       the desired nucleic acid sequence.

BACKGROUND OF THE INVENTION

For diagnostic applications in particular,  
the target nucleic acid sequence may be only a minute  
portion of the total pool of DNA or RNA in a sample to  
20       be screened, so that it may be difficult to detect the  
presence of the target nucleic acid sequence using  
nonisotopically labeled or end-labeled oligonucleotide  
probes. Thus, diagnostic tests employing DNA probes to  
detect rare species of nucleic acids are often not  
25       sensitive enough to be practical for use outside of the  
research laboratory.

-2-

One attempt to overcome the sensitivity problem is the polymerase chain reaction (PCR) method, described in U.S. Patent Nos. 4,683,195 and 4,683,202 ("the '195 and '202 patents"). This method proceeds basically, as follows:

- a) treating a sample suspected of containing the target nucleic acid sequence of interest with one oligonucleotide primer for each strand of the target nucleic acid sequence, under hybridizing conditions and in the presence of a polymerase, e.g., the Klenow fragments of Escherichia coli DNA polymerase-I, such that an extension product of each primer is synthesized if the target nucleic acid sequence is present;
- b) placing the sample after step (a) under denaturing conditions to separate any primer extension products that were synthesized from the templates on which they were synthesized to produce single-stranded molecules;
- c) treating the single-stranded molecules generated from step (b) with the primers of step (a) under the conditions of step (a), such that the new primer extension products are synthesized using both the original target sequences and the primer extension products produced in step (a) as templates, thus resulting in the amplification of the target nucleic acid sequence.

Steps (a)-(c) may be conducted sequentially or simultaneously. In addition, steps (b) and (c) may be repeated until the desired level of sequence

-3-

amplification is obtained. As discussed in U.S. Patent Nos. 4,683,195 and 4,683,202, the product of step (c) may be detected using probes.

5 The PCR method has a disadvantage in that it fails to completely overcome the sensitivity problem. The PCR method uses all four nucleotide bases to extend the primer fragments. Therefore, extension products may be created from other, non-target nucleic acid  
10 templates that may be present in the sample such as nicked, double-stranded DNA. The use of the PCR method results in considerable background of amplified DNA other than the target sequence(s).

As will be discussed in detail later, the present invention uses at least two oligonucleotides  
15 for each strand of target nucleic acid sequence and uses fewer than all four bases, thus reducing the problem of nonspecific, background amplification for a number of reasons. For example, when labeled  
20 nucleotides are used, the gap will be filled with labeled nucleotides if the nucleic acid target sequence exists in the sample and the irrelevant sequences will not be copied or labeled.

The polymerase chain reaction method also requires heat stable enzymes for the process to be  
25 automated, while the process of the present invention can be performed using heat-labile enzymes or without any enzymes, depending upon the particular embodiment. In addition, the detection of amplified nucleic acids produced in the PCR method often requires the use of  
30 gels or a capturing system, which are laborious detection methods. In contrast, the detection of the amplified sequences in the present invention is

-4-

relatively simple. For example, a Sephadex column can be used to separate the joined, oligonucleotide products formed when the target sequence is present apart from the individual nucleotides.

5 Other methods, beside the PCR method, exist for producing nucleic acids in large amounts from initially small amounts. For example, there is the method of subcloning a nucleic acid in the appropriate host system, where the desired nucleic acid is inserted  
10 into an appropriate vector which is used to transform the host. When the host is cultured, the vector is replicated, and hence more copies of the desired nucleic acid are produced. For a brief description of subcloning nucleic acid fragments, see Maniatis, T., et  
15 al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, pp. 390-401 (1982). See also the techniques described in U.S. Patent Nos. 4,416,988 and 4,403,036.

20 Other methods for synthesizing nucleic acids include the organic synthesis of a nucleic acid from nucleotide derivatives such as the methods described in U.S. Patent No. 4,356,270. Another example of the method for synthesizing nucleic acid is provided in  
25 U.S. Patent No. 4,293,652, which is a hybrid of organic synthesis and molecular cloning. The discussion of these and other methods in the '195 and '202 patents, as well as the patents listed above, are incorporated herein by reference.

#### SUMMARY OF THE INVENTION

30 The present invention relates to a process for amplifying one or more specific target nucleic acid sequences present in a sample.

-5-

5 In one embodiment of the present invention, the amplification is accomplished by using two or more oligonucleotide complement pairs wherein at least one strand of these oligonucleotide pairs has a nucleotide sequence complementary to at least a portion of the target sequence. The oligonucleotides are selected so there is a gap of at least one base when the complementary strands of the "oligonucleotide complement pairs" and the target nucleotide are hybridized to one another. The gap between the oligonucleotide complement sequences is filled by a mixture of polymerase and ligase producing an oligonucleotide repair product. The resulting mixture of hybridized molecules is then placed under denaturing conditions.

10 After the strands separate during denaturation, the ligated oligonucleotide product can hybridize to its complementary strands from other oligonucleotide complement pairs, and then the gap is filled again. The process is repeated as often as is necessary to produce the desired amount of oligonucleotide repair product. In one embodiment of the present invention, the enzymes are immobilized on a polymeric support.

25 The present method is especially useful, for amplifying sequences indicative of a genetic disorder and rare species of nucleic acid present in a mixture of nucleic acids, and further permits the effective detection of such nucleic acid sequences. The present invention provides a process for amplifying at least one specific nucleic acid sequence in a sample of a nucleic acid or a mixture of nucleic acids. Each

-6-

nucleic acid target could consist of one strand (RNA) or two separate complementary strands (DNA) of equal or unequal length.

The process is accomplished as follows:

- 5           a) Treating the sample with oligonucleotide complement pairs A,A' and B,B' under hybridizing and gap filling conditions. A is an oligonucleotide and A' is an oligonucleotide that is complementary to A. B is an oligonucleotide and B' is an oligonucleotide that is complementary to B. When the set of two pairs A,A' and B,B' are hybridized to nucleic acid target, A and B, which hybridize to one strand of the target forms a gap of one or more bases between them. Also, A' and B' form a gap of one or more bases between them. The gap is filled with labeled or unlabeled base(s) such that A and B become one strand with a continuous base sequence with one or more extra labeled or unlabeled bases, (A-Q-B), where Q is(are) the base(s) that fill the gap. Q could also be a base modified to be resistant to degradation caused by the 3'-->5'exonuclease activity of polymerases used to fill the gap. Also, A' and B' are now one strand with a continuous base sequence with one or more extra labeled or unlabeled bases, (A'-Q'-B'), where Q' is(are) the base(s) that fill the gap. Similarly, Q' could also be a modified bases resistant to degradation caused by the 3', 5'exonuclease activity of polymerases.
- 10
- 15
- 20
- 25
- 30



-7-

For any continuous base sequence A-Q-B or A'-Q'-B', Q or Q' can be composed of only one set of base pairs for any specific sequence, i.e., A-T, A-U, G-C, or derivatives of these bases. A-Q-B and A'-Q'-B' are now joined, oligonucleotide products and can now serve as "target" sequences for other oligonucleotide complement pairs. When the joined, oligonucleotide product is formed by this gap-filling, ligated process it is termed an "oligonucleotide repair product."

- b) Treating the sample under denaturing conditions to separate the oligonucleotide repair products from their targets, if the nucleic acid target sequence(s) is(are) present.
- c) Treating the sample as in step (a) with oligonucleotide complement pairs A,A' and B,B' under hybridizing and gap-filling conditions such that an oligonucleotide repair product is obtained using each of the single strands produced in step (b), resulting in the amplification of the specific nucleic acid target sequence(s) if present.

The steps may be conducted sequentially or simultaneously. In addition, steps (b) and (c) may be repeated until the desired level of sequence amplification is obtained.

In another embodiment of the present invention, photosensitive molecules, x and y, are attached to each strand of the oligonucleotide

-8-

complement pairs at the ends of the molecules which are to be joined together. X and y are compounds capable of forming carbon-carbon double bonds and undergoing [2 + 2] photocyclodimerization, thereby linking the oligonucleotide products when photoactivated and forming a joined, oligonucleotide product termed "an oligonucleotide photocyclodimerized product." In this embodiment, a gap between contiguous nucleotide strands is unnecessary. A gap of one or two bases is permissible.

This invention is also related to methods for the detection of the amplified specific nucleic acid sequence and diagnostic kits applicable thereto.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates a 48 base pair length sequence of HIV from GAG region desired to be amplified. The base pairs which fill the gap is depicted above and below the 48 base pair.

Figure 2 shows a 12% polyacrylamide-7M urea gel, where each lane consists of a cycle that includes: boiling the mixture of human immunodeficiency virus (HIV) plasmid target with labeled oligonucleotides, attached to photoreactive compounds, quick cooling the mixture, to 37°C and irradiating the mixture for five minutes.

Fig. 3 depicts an apparatus for carrying out one embodiment of the method of the present invention wherein the enzymes used are immobilized on a polymeric support.

-9-

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTSDefinitions

The term "oligonucleotide" as used herein, is defined as a molecule comprised of three or more deoxyribonucleotides or ribonucleotides, preferably more than five.

The term "blunt end" is defined as two oligonucleotides that have sequences complementary to each other and at least one end of equal length when hybridized together.

"Sticky end" as defined in this application refers to two oligonucleotides that have sequences complementary to each other and at least one end of unequal length when hybridized together. The categories of blunt end and sticky end are illustrated below.

The term "two oligonucleotide complement pairs" are at least four different oligonucleotides which are designated, for example, A,A' and B,B' wherein oligonucleotide A has a base sequence complementary to A', and oligonucleotide B has a base sequence complementary to B'. Each pair could be equal or unequal in length, which is illustrated as follows:

Category 1. Oligonucleotide Complement Pairs with Blunt Ends

5' ——— A ——— ATC-3'  
3' ——— ——— TAG-5'  
A'

5' -CCC ——— B ——— 3'  
3' -GGG ——— ——— 5'  
B'

-10-

Category 2. Oligonucleotide Complement Pairs Each Having One Blunt and One Sticky End



Two oligonucleotide complement pairs with a sticky end on one end of each pair are preferred as the bases for forming the collection of amplified oligonucleotides of the present invention. It would be understood that more than two oligonucleotide pairs could be used in the process of the present invention.

The term "gap" as used herein refers to the absence of one or more bases between A and B, E and F, or between A' and B', E' and F'. If more than two pairs are used then there may be more than one gap. The gap is created when the pairs are hybridized to the nucleic acid target, as for example:

Line 1: 5' ————— ATCATATCCC ————— 3'

Line 2: 3' ————— TAG GGG ————— 5'

(the gap)

Line 1 is the sequence of the nucleic acid target, and line 2 represents oligonucleotide complements A' and B', as hybridized to the nucleic acid target sequence. If the target sequence is double-stranded, oligonucleotides A and B will form a hybrid with the sequence complementary to the target sequence shown above in line 1.

A. GAP-FILLING, LIGATION AMPLIFICATION

In one embodiment of the process for amplifying nucleic acid sequences of the present invention, at least two oligonucleotide complement pairs are combined with the sample suspected or known

-11-

5 to contain the target nucleic acid sequence of interest  
under hybridizing conditions. The oligonucleotide  
complement pairs are selected so that there is a gap in  
the nucleotide sequence of at least one base between  
10 the two complements when the two complements are  
hybridized with the nucleic acid target sequence. The  
gap is then filled and the two oligonucleotides are  
ligated together, producing an oligonucleotide repair  
product. The process of gap-filling plus ligation is  
15 analogous to the repair of mismatched bases, and other  
errors that occur during DNA replication, repair of UV  
damage, and other processes in vivo. The nucleic acid  
target sequence and the oligonucleotide repair product  
sequence may then be separated and the process repeated  
20 over and over again until the desired level of  
amplification has been achieved. To avoid a problem of  
background synthesis occurring during the gap filling  
step, the two or more oligonucleotide complement pairs  
are selected so that the gaps between them will require  
25 less than all four bases to fill in the gap, preferably  
one set of complementary bases, namely A-T, A-U, or  
G-C. Without all four bases, random synthesis would  
not be initiated by nucleic acid sequences that may  
have nicks or are in the process of replication or  
transcription.

#### 1. Nucleic Acid Target Sequences

30 The process of the present invention can  
produce exponential quantities at least one specific  
nucleic acid sequence relative to the number of  
reaction steps involved, provided that (a) at least  
part of the nucleic acid target sequence is known in

-12-

sufficient detail that oligonucleotide pairs can be synthesized which can hybridize to it, or (b) the target sequence can be isolated in large enough quantities to produce enough oligonucleotide complement pairs for use in the process. Any source of nucleic acid can be utilized as the source of the target nucleic acid sequence, in purified or nonpurified form. For example, the process may employ either single-stranded or double-stranded DNA or RNA. In addition, a DNA-RNA hybrid which contains one strand of each may be utilized. A mixture of any these nucleic acids may be employed. The specific nucleic acid sequence to be amplified may be only a fraction of a larger molecule. It may be a minor fraction of a complex mixture, such as a portion of the HIV (human immunodeficiency virus) gene integrated in the genomic DNA of an infected person, or bacterial nucleic acid present in very low quantities in a particular biological sample.

To determine the sequence of the target sequence(s), or as a sample to be tested, the nucleic acid or acids of interest may be obtained from any source, for example, DNA or RNA, isolated from bacteria, viruses, yeast and higher organisms such as plants or animals, from plasmids such as PBR 322 and M13, from cloned DNA or RNA by a variety of techniques known to those skilled in the art. DNA may also be extracted from cells grown in tissue culture by techniques such as those described by Maniatis et al., Molecular Cloning, a Laboratory Manual (New York: Cold Spring Harbor Laboratory, 1982), pp.280-281.

-13-

## 2. Oligonucleotide Complement Pairs

The oligonucleotide complement pairs are preferably oligodeoxyribonucleotides. In addition, the pairs must be long enough to hybridize to the nucleic acid target sequence(s). The length of the complement pairs can vary from four bases to hundreds of bases. A short oligonucleotide generally requires cooler temperatures to form stable, hybrid complexes. The oligonucleotide sequences synthesized are selected so that two oligonucleotide complement pairs would both hybridize to the target sequence and yet leave a gap of one or more bases. If the target sequence is single-stranded, only one half of each oligonucleotide pair would hybridize to the target. More than two pairs of oligonucleotide complements can be employed in the process of the invention as long as the amplification will remain specific for the nucleic acid target sequence(s).

The oligonucleotide complement pairs may be prepared using any suitable method, e.g., phosphoramidites (Applied Biosystems Inc.) may be used as starting materials and may be synthesized as described by Beaucage, et al., Tetrahedron Letters, 22, 1859-62 (1981) and phosphorylated at 5'-end by methods well-known in the art.

## 3. Denaturation

The strand separation can be accomplished by any suitable denaturing method including physical, chemical or enzymatic means. One physical method of separating the strands of the nucleic acid involves heating the nucleic acid until it is completely (more

-14-

than 99%) denatured. Typical heat denaturation may involve temperatures ranging from about 80°C to 105°C, for times ranging from about 1 to 10 minutes. Strand separation may also be induced by an enzyme from the class of enzymes known as helicases or the enzyme RecA, which has helicase activity and in the presence of riboATP is known to denature DNA. The reaction conditions suitable for separating the strands of nucleic acids with helicases are described by Cold Spring Harbor Symposia on Quantitative Biology, Vol. XLIII "DNA Replication and Recombination" (New York: Cold Spring Harbor Laboratory, 1978), B. Kuhn et al., "DNA Helicases", pp. 63-67, and techniques for using RecA are reviewed in C. Radding, *Ann. Rev. Genetics*, 16:405-37 (1982).

#### 4. Gap-Filling/Ligation Steps

Generally, the gap-filling and ligation steps occur in a buffered aqueous solution, preferably at a pH of 7-9, most preferably at a pH 7.5. The oligonucleotide complement pairs will be present in molar excess of about  $10^5$ - $10^{15}$  pairs per nucleic acid target sequence. The exact amount of the pairs to be used in diagnostic purposes may not be known due to uncertainty as to the amount of the nucleic acid target in a sample. However, using an average amount of  $10^{15}$  oligonucleotide complement pairs is applicable in a typical diagnosis assay format. A large molar excess is preferred in any case to improve the efficiency of the process of the invention.

In the process of the invention, typically only two complementary deoxyribonucleoside



-15-

triphosphates would be used in the gap-filling step, dATP and TTP, or alternatively, dCTP and dGTP. Sticky-ended oligonucleotide complement pairs can be selected, so that only one type of deoxyribonucleoside triphosphate is required to fill in any gaps. Sticky-ended complement pairs can also be selected so that two complementary nucleotides and one other nucleotide are used to fill the gap.

It is preferred to utilize modified nucleoside triphosphates known in the art to be resistant to the exonuclease activity of polymerases, as described by D. Shortle et al., Proc. Natl. Acad. Sci. USA, 79: 1588-92 (1982); T. A. Kunkel, *ibid.*, 78: 6734-38, (1981); F.R. Bryant et al., Biochemistry 18: 2825-28 (1979). Such molecules could be thymidine 5'-O-(1-thiotriphosphate) (TTP [ $\alpha$ S]), 2'-deoxyadenosine 5'-O-(1-thiotriphosphate) (dATP [ $\alpha$ S]) and the thio-derivatives of deoxycytidine, deoxyguanine, deoxyuridine and of cytidine, guanidine, adenosine, thymidine, and uracil. Other derivatives of these bases that are resistant to nuclease activity are suitable, such as  $\alpha$ -imido derivative of triphosphate bases.

Sufficient deoxyribonucleotide triphosphates are added to the gap-filling/ligating mixture in adequate amounts and the resulting solution is heated to about 90°C to 100°C for approximately one to five minutes, preferably from one to three minutes. The solution is then allowed to cool to room temperature to allow hybridization to occur. To the cooled solution, appropriate catalysts are added to induce the filling and sealing of the gap under conditions known in the

-16-

art. For example, known DNA polymerases can be used for the gap-filling and known DNA ligases can join the resulting product after the polymerase has filled the gap. The gap-filling ligation process may occur at  
5 from 4°C up to a temperature at which the catalytic agents no longer function efficiently; the temperature is generally no greater than about 40°C. Thus, for example, if T4-DNA polymerase and T4-DNA ligase are used, the temperature is generally no greater than  
10 about 40°C (most conveniently, the reaction occurs at room temperature or even at 4°C). If heat insensitive enzymes are used, then the process could occur at the melting temperature of the hybrids templates.

The catalytic agent may be any compound or  
15 system which will function to fill the gap between the two or more oligonucleotides hybridized to the nucleic acid target. Enzymes suitable for this purpose include E. coli DNA polymerase-I, Klenow fragment of E. coli DNA polymerase-I, T4-DNA polymerase, reverse  
20 transcriptase for adding bases to fill in the gap, T4-DNA ligase to join the oligonucleotide(s), plus nucleotides added by the polymerase, to the other oligonucleotide complements, forming a joined oligonucleotide product, which in this embodiment is  
25 termed the oligonucleotide repair product.

The oligonucleotide repair products hybridized to the nucleic acid targets are in a double-stranded form. In the next step, the strands of the double-stranded molecule are again separated as  
30 described above to provide single-stranded molecules.

In order to generate a mode of amplification, the joined oligonucleotide molecules can hybridize to

-17-

two more oligonucleotide complement pairs. If necessary, additional enzymes, appropriate deoxyribonucleotide triphosphates, and oligonucleotides may be added for the reaction to proceed.

5           The steps of strand separation, gap-filling and ligation can be repeated as often as needed to produce the desired amount of the specific joined, oligonucleotide product assuming that one copy of  
10           single-stranded nucleic acid target sequence was present in the mixture at the beginning. As long as these steps will be repeated, the amplification of the specific nucleic acid target sequence will take place in an exponential way. This process could be used to  
15           amplify other nucleic acid target sequences, by adding different oligonucleotide complement pairs (or triplets, etc.) that hybridize to different specific nucleic acid target sequences without changing other conditions involved.

20           This particular embodiment of the invention could be performed in a stepwise fashion, in which new reagents are added after each step, or simultaneously, in which all reagents are added at once. The reagents can also be added after a given stage. Each step of  
25           the embodiment using heat-stable enzymes will occur sequentially regardless of the initial concentration of all the reagents. When heat-sensitive enzymes are used, it is necessary to add the gap-filling and sealing agents after every strand separation (denaturation) step.

30           In one embodiment of the present invention, the catalytic agents are immobilized on polymeric supports. After the gap-filling and ligation step, the

-18-

products can be eluted from the immobilized enzymes and denatured to single strands. At this point, the nucleic acid target sequence(s) and joined, oligonucleotide product(s) are available to form hybrids with additional, oligonucleotide complement pairs. These new hybrids are then transferred back to the immobilized enzymes to form joined products. The steps of the reaction may be carried out stepwise or simultaneously and may be repeated as often as desired.

For smaller oligonucleotide complements, for example, 6-10 bases, heat-sensitive enzymes could be employed in a simultaneous procedure, since the melting temperature of double-stranded oligonucleotides of this range is around 40°C, and heat-sensitive enzymes are active at this temperature.

If heat-stable gap-filling and sealing agents are used, such as thermostable polymerase and ligase, then the process could be employed at an elevated temperature, preferably 60-90°C depending on the heat-stable enzymes. The temperature would also be determined by the temperature at which there will be an equilibrium between single and double-stranded nucleic acids. Such a heat-stable polymerase is described by A.D. Kaledin, et al., *Biokhimiya*, 45, 644-51 (1980). A heat-stable polymerase and ligase could be extracted from Thermus thermophilus.

After the appropriate period of performing the process has passed, and the desired amount of the oligonucleotide repair product has accumulated, the reaction may be stopped by inactivating the enzymes in any known manner or separating the components of the reaction on spun or Sephadex columns, by filtration, or by gel electrophoresis as known in the art.

-19-

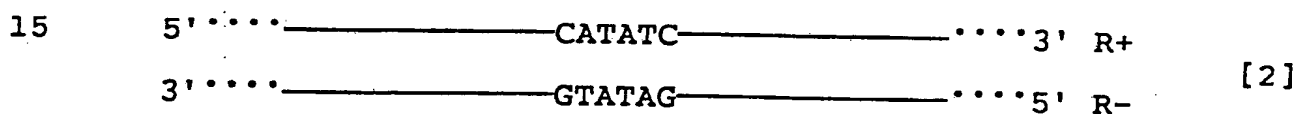
The process of this invention may be employed as an automated process. To do this, the reaction is cycled through a denaturing step, a reagent addition step, and a reaction step. Also the process may be conducted continuously by adding the reagents automatically by a pump after the denaturing step, while the heating-cooling step could be performed by a specially designed, controlled heater block.

The present invention is demonstrated diagrammatically below. Using two oligonucleotide complement pairs that have one sticky end each (Category 2.)

Two Sticky-Ended Pairs



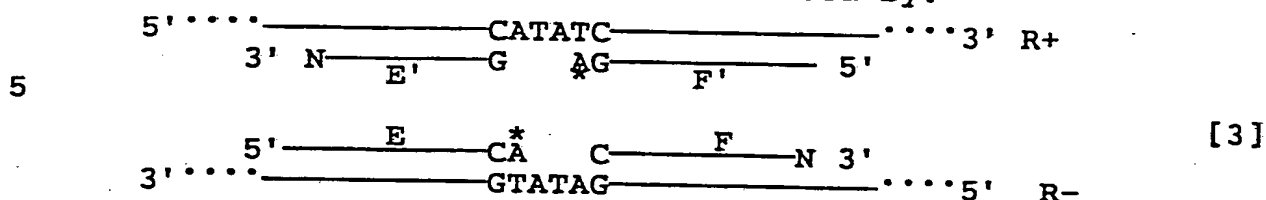
N is a nucleotide modified to protect the strands from degradation by 3' exonucleases, such as that found as part of T4-DNA polymerase. Oligonucleotide complement pairs E, E' and F, F' are selected or synthesized to correspond to sections of the complete nucleic acid target sequence R. R is a double-stranded DNA comprising complementary strand R+ and R- represented as:



When [R] is mixed with a molar excess oligonucleotides of complement pairs E, E' and F, F' under conditions where the double-stranded molecules are denatured and then are permitted to rehybridize, R+ forms stable,

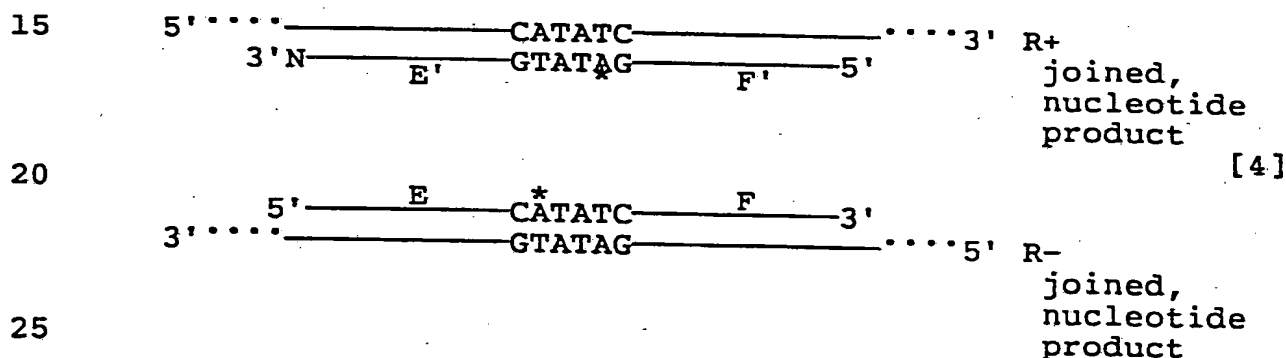
-20-

double-stranded hybrids with E' and F', while R- forms hybrids with E and F as illustrated by:



\*  
 A represents the modified nucleotide N of diagram [1] such as [NTP's αS].

Next, TTP, dATP, E. coli. Klenow fragment and T4-DNA ligase are added to mixture, permitting the gap in the double strands to be filled and sealed, producing:



In this particular example, the Klenow fragment adds the nucleotides to the 3' of F' and the ligase joins the added nucleotides to the 5' end of E'. E and F are joined in the same fashion.

30 In the first cycle, two new single-stranded DNA have been produced. After the next denaturing step, the molar excess of the two oligonucleotide complement pairs can hybridize to the original nucleic acid targets as well as to the oligonucleotide repair product formed in the first cycle, thus four more  
 35

-21-

oligonucleotide repair products are formed in the second cycle. The formation of the new joined oligonucleotides product will proceed exponentially.

5 In this process, dATP and TTP could be labeled with radioactive labels, such as  $^{32}\text{P}$ ,  $^{35}\text{S}$ , or  $^{125}\text{I}$ . Non-radioactive labels can also be used. For example, TTP could be replaced by Biotin-dUTP (Enzo Biochem), and dATP could be replaced by Biotin-dATP as described by G. Gebeyehu et al., Nucleic Acid Res. 15, 10 4513-34 (1987).

15 In the case when blunt-ended oligonucleotide pairs in solution are used (category 1), blunt-end ligation could occur between the set of two pairs in solution. However, since only the bases which filled the gap are labeled, the blunt end ligation products will not be counted in the detection step.

#### B. PHOTOCHEMICAL LIGATION

20 In another embodiment of the present invention, photosensitive molecules, x and y, that can form carbon - carbon double bonds and undergo [2 + 2] photocyclodimerization are employed to link the oligonucleotide complements together. The photosensitive molecules x and y are attached to each strand of the oligonucleotide complement pairs at the ends of the molecules which are to be joined together. 25 X (or X') and y (or y') can be the same molecules or different ones, as long as the photocyclodimerization reaction can occur. This embodiment of the invention can be illustrated as:

$$\begin{array}{ccc} 5' & \text{I} & \text{ATCAT-x } 3' \\ 3' & \text{I}' & \text{TAGTA-x' } 5' \end{array} \qquad \begin{array}{ccc} 5' \text{y} & \text{TCCC} & \text{J} & 3' \\ 3' \text{y}' & \text{-AGGG} & \text{J}' & 5' \end{array}$$

Line 3: 5' \_\_\_\_\_ ATCATATCCC \_\_\_\_\_ 3'  
 4: 3' \_\_\_\_\_ TAGTA AGGG \_\_\_\_\_ 5'

I'                  x<sup>+</sup>y'                  J'

Line 3 represent the sequence of the nucleic acid target, and line 4 represent oligonucleotides I' and J' hybridized to the nucleic acid target sequence. If the target sequence is double-stranded, oligonucleotides I and J will form a hybrid with the sequence complementary to the target sequence shown in line 3. After the hybrids with the target sequence is formed, x' and y' are photoactivated, as will be described in detail later, producing a photodimer, thereby joining oligonucleotides I' and J' together.

In order to create such bonds, these photoactivatable groups should be in close proximity to each other (4Å separation) and nearly parallel, thus permitting excellent pi-electron overlap. This proximity provides strong attractive interaction when one of the molecules is excited, Schmidt et al., Pure Appl. Chem., 27: 647-78 (1971). Without the close proximity, the photoactivable molecules are too far apart and/or unsuitably oriented to form the double bonds necessary to create stereoisomeric photodimers. Consequently, no photochemical ligation between oligonucleotide complements pairs in solution are likely to occur. Rather, the photoactivatable groups, x and y, will only be close enough to each other to bond together when the complements are hybridized to the nucleic acid target sequence or to a joined, oligonucleotide target created in a previous cycle.



-23-

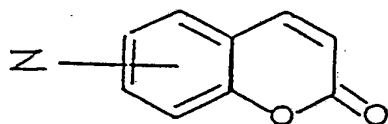
Photosensitive molecules, x and y, that can form carbon-carbon double bonds and undergo [2+2] photocyclodimerization are useful in this embodiment of the present invention. These photosensitive molecules can include: cinnamic acids, M.D. Cohen et al., J. Chem. Soc., 2000-13 (1964); T. Ishigami, et al., Bull. Chem. Soc. Japan, 49: 3578-83 (1976); styrene derivatives, A. L. Elgavi, et al., Thesis, Weizmann Institute of Science, Rehovot, Israel (1974); stilbenes, M.D. Cohen, et al., J. Chem. Phys. Lett. 7: 486-90 (1970); aliphatic mono-, di- and triene dicarboxylic acid derivatives, T. J. Sadeh, Am. Chem. Soc. 84: 3970 (1962); M.J. Lahav, Chem. Soc. B., 312-17 (1967); M. Lahav, Tetrahedron Lett. 2957-62 (1966), cross-conjugated aromatics, B. S. Green, Tetrahedron Lett., 4249-52 (1970); benzoquinones, D. Rabinovich, et al., J. Chem Soc. B., 144-49 (1967); allenes, Z. Berkovitch-Yellin, et al., J. Chem. Soc. Chem. Comm., 178-79 (1982); and psoralenes. In other words, x and y are any molecules that are capable of absorbing light and thereby creating cyclobutane derivatives.

The x and y moieties are preferably aminopsoralenes or coumarines that have a tendency to form a  $\pi$  complex that overlaps and therefore facilitates the [2+2] photocyclodimerization. Moreover, psoralenes are photoactivated at 320-460 nm wavelength, which is a wavelength that does not damage the nucleic acids in the sample. The oligonucleotide complements must be sufficiently long enough to form a stable hybrid with the target in order to reach the proper proximity and the maximum pi-electron overlap between x and y. As used herein, the term "light source" refers

-24-

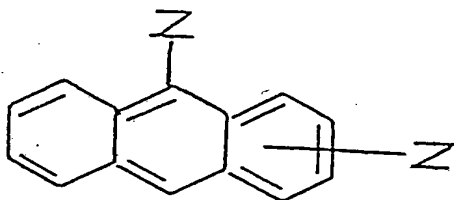
to an irradiation source that could excite  $\pi \rightarrow \pi^*$ ,  
 $n \rightarrow \pi^*$  in the range of 200 nm - 500 nm, preferably in  
the range of 300 nm - 400 nm, where the source is near  
U.V. light or ultraviolet laser pulses. The creation  
of the new bond, by using a light source, takes place  
in a buffered aqueous solution, at a pH of 7-9,  
preferably at pH 8.

Particularly, preferred photoactivatable  
molecules, x and y, for use in the process of the  
present invention are the following:



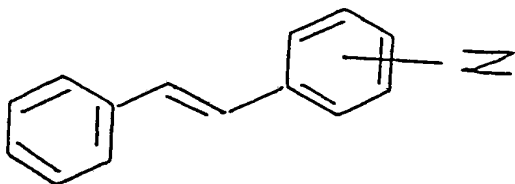
Coumarin derivatives

where Z is H, methoxy, acetoxy,  $C_1-C_5$  alkyl, halogen  
and di- or tri- derivatives of these groups;



Anthracene derivatives

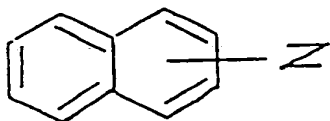
where Z is H, CN, alkyl or halogen;



Stilbene derivatives

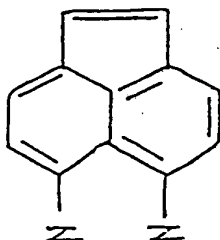
where Z is H, CN, alkyl, halogen,  $CO_2H$ , or alkyl ester;

-25-



Naphthalene derivatives

where Z is H, CN, -O-Alkyl, halogen or carboxy;



Acenaphthylene derivatives

where Z is H, CN, alkyl, halogen, or carboxy.

5           The photoreactive molecules may be attached to the oligonucleotides at the 5' and 3' ends using any of the following methods:

10           a) Modification of 5'-hydroxyl group by amines as described by L.M. Smith et al., Nucleic Acid Res. 13: 2399 (1985), by 5'-thiols as described by B.S. Sproat et al., Nucleic Acid Res., 15: 4837 (1987), by phosphate group using T4-DNA ligase, by chemical phosphorylation as described by T. Horn et al., Tetrahedron Lett. 27: 4705 (1986), or by other methods of modifications.

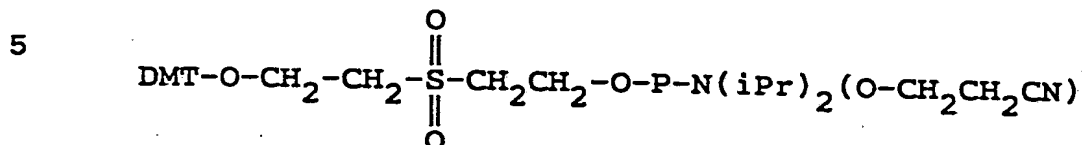
15           b) Modification of 3'-hydroxyl group by amino group is described by U.S. Patent 4,128,639. A 3'-phosphate group could be attached to the oligonucleotide using the same chemical phosphorylating agent, as described above by T. Horn et al.

20           The preferred method of attaching the photoreactive molecule to the 3' and 5' ends of the oligonucleotides is by attaching phosphate groups to the desired 3' and 5'-hydroxyl groups, e.g., the 3'-phosphate group is synthesized as follows: controlled

25

-26-

pore glass (500Å) (PIERCE) that is modified with long chain alkyl amine is allowed to condense with a phosphorylating agent,



10 (Glen Corp.) (DMT is dimethoxytrityl; iPr is isopropyl), in the presence of tetrazol at ambient temperature. The phosphate group is then oxidized using an oxidizing agent, preferably iodine solution, at room temperature. After detritylation, using a mild acid, preferably dichloroacetic acid in

15 dichloromethane, a hydroxyl group is exposed and the desired oligonucleotides could be synthesized on this hydroxyl group, as known in the art.

The 5'-phosphate group, attached to the desired oligonucleotides, could be prepared by ligation procedures under conditions known in the art. The 3' or 5'- end phosphate groups could be activated with activating agents, preferably imidazole, using a condensing agent, preferably water-soluble carbodiimide, at ambient pH, preferably pH 6.0, at room

20

25 temperature.

The aminophotoreactive group is reacted with activated phosphate group at pH 7.8 as described by B.C.F. Chu et al. in Nucleic Acid Res., 11: 6513-29 (1983).

30

#### C. DETECTION OF THE JOINED, OLIGONUCLEOTIDE PRODUCT

The joined, oligonucleotide product can be detected by any number of methods for detecting labeled

-27-

molecules and/or molecules having a particular length or sequence. For instance, the reaction mix may be passed through a Sephadex column to separate the labeled NTPs and the joined oligonucleotide products. Since individual nucleotides are much smaller than the length of the two or more oligonucleotide complement pairs, the detection of amplified material should be fairly simple based on size alone.

Another technique to detect amplified sequence would require the construction or isolation of probes that share complementary sequences with enough of each and every oligonucleotide complement pair to bind and hold the joined product preferentially. Such probes can be immobilized on any suitable substrate. They may also be labeled differentially if desired.

The present invention may be used for in vitro diagnostics. The process of amplifying nucleic acid sequences enables the detection of specific nucleic acid sequences associated with infectious disease, genetic disorders or cellular disorders such as cancer. Amplification is particularly useful when the amount of nucleic acid target available for diagnosis is in minute quantities, as, for example, in the prenatal diagnosis of sickle cell anemia, which requires obtaining DNA from fetal cells. Furthermore, it is within the ability of those skilled in the art, that the length and sequences of the oligonucleotide complements can be varied to detect deletions and/or mutations in genomic DNA from any organisms. These small changes are important in the diagnosis of such conditions as cystic fibrosis,  $\alpha$ -thalassemia,  $\beta$ -thalassemia and the like.

-28-

5           The process of the invention may also indicate the presence of various agent like pathogenic viruses including human immunodeficiency virus (HIV), herpes, hepatitis B and bacteria including Salmonella and Chlamydia.

10           U.S. Pat No. 4,358,535, issued to Falkow, describes the use of specific DNA hybridization probes for the diagnosis of infectious diseases. A problem inherent in the Falkow procedure is that a relatively small number of pathogenic organisms may be present in a clinical sample from an infected patient and the DNA extracted from the pathogens may constitute only a very small fraction of the total DNA in the sample. Specific amplification of suspected sequences in a sample prior to the hybridization to the probes and the immobilization on filters samples could greatly improve the sensitivity and specificity of these procedures.

15           In a further embodiment, the amplified species could be detected by a simple method as follows: A capture oligonucleotide probe that has a nucleic acid sequence which is complementary to both E and F or E' and F' or to each one of them separately could be used to capture the amplified labeled products.

20           In another embodiment, the deoxyribonucleotide triphosphates involved in the process could be radioactively labeled such as with  $^{32}\text{P}$ ,  $^{35}\text{S}$ ,  $^{125}\text{I}$  and others with non-radioactive labeling.

30           Another means to facilitate the clinical use of DNA probes for the diagnosis of infectious diseases is the substitution of non-radioactive labels for

-29-

radioactive ones. As described in EP 63,879 to Ward, biotin-containing DNA probes are detected by chromogenic enzymes linked to avidin or biotin-specific antibodies. This type of detection is convenient, but relatively insensitive. The combination of DNA amplification by the present method and the use of Bio-dUTP to label the bases that filled the gap could provide the convenience and sensitivity required to prepare useful diagnostic kits and to overcome the difficulties associated with both Falkow and Ward procedures when these techniques are applied in a routine clinical setting.

The use of the Falkow and Ward methods, the synthesis of oligonucleotides, the calculation of the number of sequences amplified per cycle, and other matters that pertain generally to amplification of nucleic acid sequences are described in the '195 and '202 applications and these applications are incorporated herein by reference.

The invention will now be illustrated by examples. The examples are not intended to limit the scope of the present invention. In conjunction with the general and detailed description above, the examples provide further understanding of the present invention and outlines some aspects of the preferred embodiments of the invention.

#### EXAMPLE 1

The desired sequence to be amplified using the gap-filling/ligating embodiment of joining the oligonucleotides is a 48 base pair sequence that coded for HIV at GAG region 2106-2153. This sequence is

-30-

chosen for its G-C content which is about 52% and has the following sequence:

5'-TCCTACAAGGGAAGGCCAGGGAATTTCTTCAGAGCAGACCAGAGCC-3'  
3'-GGATGTTCCCTTCCGGTCCCTTAAAAGAAGTCTCGTCTGGTCTCGGT-5'

5 The following four oligodeoxyribonucleotides are examples of the many blunted-ended sequences that can be used for amplification according to the method of the invention.

10 A - 5'-TCCTACAAGGGAAGGCCAGGG-3'  
A' - 3'-GGATGTTCCCTTCCGGTCCCT-5'  
B - 5'-TCTTCAGAGCAGACCAGAGCC-3'  
B' - 3'-GAAGTCTCGTCTGGTCTCGGT-5'

15 The blunt ends are created after incorporation of 2'-deoxyadenosine 5'-0-(1-thiotriphosphate) (dATP[αS]) at the 3' end of each sequence. A gap of 5 base pair exists between the oligonucleotides A and B or A' and B' when these strands are hybridized to their complementary strand of the target sequence shown above.

20 Similarly, the following four oligodeoxyribonucleotides are suitable sticky-ended sequences that may be utilized in the process of the invention.

25 E - 5'-TCCTACAAGGGAAGGCCAGGGA-3' 22 bases  
E' - 3'-GGATGTTCCCTTCCGGTCCCTTA-5' 22 bases  
F - 5'-TCTTCAGAGCAGACCAGAGCC-3' 21 bases  
F' - 3'-GAAGTCTCGTCTGGTCTCGGT-5' 21 bases

30 All of oligodeoxribonucleotides described above are synthesized and purified by the following procedure.

#### I. Automated Synthesis Procedures.

The 2-cyanoethyl phosphoramidites are purchased from Applied Biosystems Inc. The procedure includes



-31-

condensation of nucleoside phosphoramidites to a nucleoside-derivatized controlled Pore glass bead support (500Å) 30 mg, using DNA synthesizer from Applied Biosystems Inc., Type 380B-02. The cycles includes detritylation with 2% trichloroacetic acid in dichloromethane; condensation using tetrazol as an activating proton donor; capping with acetic anhydride and dimethylaminopyridine; oxidation of the phosphite to the phosphate with 0.1 M  $I_2/H_2O$ /Lutidine/Tetrahydrofuran, following detritylation using 2% trichloroacetic acid in dichloromethane. Cycle time is approximately 30 minutes. Yields at each step are essentially quantitative and are determined by collection and spectroscopic examination of the dimethoxytrityl alcohol released during detritylation.

## II. Oligodeoxyribonucleotide Deprotection And Purification Procedures

The solid support is removed from the column and exposed to 1 ml concentrated ammonium hydroxide at 60°C for 16 hours in a closed tube. Ammonia is removed and the residue is applied to a preparative 12% polyacrylamide gel using a Tris-borate buffer (pH 8) containing 7M urea. Electrophoresis is carried out at 20 volts/cm for 5 hours after which the band containing the product is identified by UV shadowing of a fluorescent plate. The band is excised and eluted with 1 ml double distilled water overnight at room temperature. This solution is filtered and the supernatant is extracted (3 x 300 microliter) with n-butanol. The water phase is placed on a Sephadex G50 column (Pharmacia) (1x 10cm). The elution is monitored

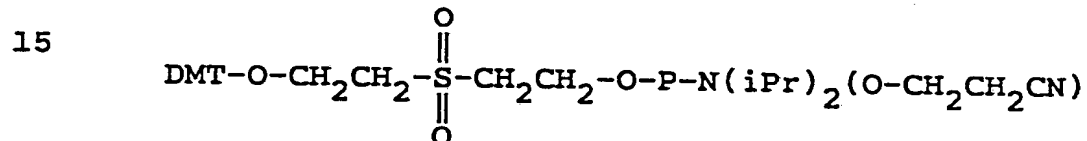
-32-

by UV absorbance at 260 nm and the appropriate fraction collected, quantitated by UV absorbance in a fixed volume and evaporated to dryness at room temperature in a vacuum centrifuge.

5      III. 5'- Phosphorylation of A',B, E'and F sequences

In order to phosphorylate 5'-ends of A', B, E' and F, a new-phosphorylating reagent as described by T. Horn et al., Tetrahedron Letters 27, 4705-08 (1986) is used. Only the ends of the nucleotides that are to be joined together will be phosphorylated.

The synthesis of A', B, E' and F is described as in I. Automated Synthesis Procedure, but after the last cycle, the phosphorylating reagent



(Glen Corp. Inc.) (DMT is dimethoxytrityl group; iPr is isopropyl) is condensed to each of the sequences A', B, E' and F using tetrazol as an activating proton donor, followed by capping with acetic anhydride and dimethylaminopyridine; oxidation of the phosphite to the phosphate with 0.1M I<sub>2</sub>/H<sub>2</sub>O/Lutidine/Tetrahydrofuran, following detritylation using 2% trichloroacetic acid in dichloromethane. Cycle time is approximately 30 minutes.

The 5'-phosphorylated A', B, E' and F are purified and quantitated by the procedure described in II above.

-33-

EXAMPLE 2

This experiment illustrates the amplification of the 48 base pair sequence of Example 1 by using a set of two blunt-ended oligonucleotide complement pairs, where a gap of 5 bases (A,T) exists between the pairs. In the first step the polymerase will incorporate dATP[ $\alpha$ S] in order to protect the complement pairs from the 3'-exonuclease activity of DNA polymerase I and to form blunt-ends.

Amplification of the 48 base pairs starts from step 2.

Step 1:

50 microliters containing 1mM MgCl<sub>2</sub>, 20mM 2-mercaptoethanol, 100 mg of bovine serum albumin per ml, 0.1mM ATP, and 0.1mM of dATP[ $\alpha$ S]) (Sigma) are added to a 60 microliter buffer solution containing 50mM Tris-HCl, pH 7.5.

To this solution are added 5 microliters of solution containing 100 picomoles each of A and B' and phosphorylated A' and B and 0.1 picomole of the 48 base pairs to be amplified. The resulting solution is heated to 100°C for 2 minutes, which allows separation of the strands and hybridization of the A, A', B, and B' to the target. Then, 4 units of Klenow fragment of E. coli DNA polymerase-I are added and the reaction is incubated for 10 minutes at room temperature.

The two pairs are now blunt-ended and protected against exonuclease activity of polymerase.

-34-

Step 2:

In order to produce and amplify the joined, oligonucleotide products, the following sub-steps are employed.

- 5 a. Add 1 nanomole each of dATP and TTP and 100 picomoles of each of  $\alpha$ -<sup>32</sup>P-dATP and  $\alpha$ -<sup>32</sup>P-TTP (Specific activity 1000 cpm/picomole).
- b. Heat for 2 minutes to 100°C.
- c. Cool to room temperature for 2 minutes.
- 10 d. Add a mixture of 4 units of Klenow fragment of E. coli DNA polymerase-I and 2 units of T4-DNA ligase.
- e. Incubate for 10 minutes at room temperature.

15 Sub-step (a) in the cycle can be omitted if a sufficient amount of nucleotide triphosphates are present initially. The cycle is repeated 22 times. 2 microliter aliquots from cycles 2, 4, 7, 10, 13, 16, 19, and 22 are applied to a 12% Polyacrylamide gel, 7M urea using 0.089M Tris-borate buffer, pH 8.3.

20 Electrophoresis is carried out at 20 volts/cm for 5 hours. The gel is exposed to sensitive film (Kodak) for 5 hours.

The resulting reaction mixture is loaded on a Sephadex G40/50 column (1X10cm) (Pharmacia) and eluted with double distilled water. Two distinct peaks are separated and monitored by a Geiger counter. The first peak, appearing after elution of 5-7 ml of water, consists of the amplified 48 base pairs, and the second peak, appearing after elution of 14-18 ml of water, consists of  $\alpha$ -<sup>32</sup>P-dATP and  $\alpha$ -<sup>32</sup>P-TTP.

25

30

In this example, steps 1 and 2 are employed separately. However, the two steps can be combined

-35-

into one step, so that the cycling could start immediately by using a mixture of dATP[ $\alpha$ S] that could be added in Step 2, sub-step (a) of the cycle, or, alternatively, the protected blunt-ended pairs could be prepared separately, purified, quantitated, and used as desired.

### EXAMPLE 3

This example illustrates a non-radioactive labeling during the amplification protocol of this invention. 100 microliters solution containing 1mM  $MgCl_2$ , 20mM 2-mercaptoethanol, 100mg of bovine serum albumin per ml and 50mM Tris-HCl(pH 7.5). 5 microliter of a solution containing one nanomole of each of A, A', B, and B' where B and A' are phosphorylated at their 5'-ends, and 10 picomole of 48 base pair as prepared in Example 1 were added. 1 nanomole of each of dATP and Bio-11-dUTP (Enzo Biochem) and 100 picomoles of dATP [ $\alpha$ S] are also added. The number 11 designated a linker arm of 11 atoms.

The resulting solution is subjected to cycles as described above e.g., the mixture is heated to 100°C for 3 minutes and allowed to cool to room temperature for 2 minutes, whereupon 4 units of Klenow-fragment of E. coli DNA polymerase-I and 2 units of T4-DNA ligase are added. The reaction mixture is incubated for 5 minutes at room temperature. The cycle is repeated 20 times as described in the previous experiment.

The reaction mixture is then diluted with 100 microliter of 2M ammonium acetate buffer, pH 7.5. The mixture is serially diluted 4-fold using 1M ammonium acetate as a diluent. Aliquots (50 microliter) of the

-36-

amplified DNA solution is added to the wells of 96-well Dynatech Immulon II microtiter plates (Dynatech). The plates are sealed and incubated for 90 minutes at 37°C to bind the amplified DNA to the plates. After the incubation, the wells are washed at room temperature twice with 200 microliters aliquots of 0.3M NaCl and 0.03M sodium citrate, pH 7.0, and once with 200 microliter of the same buffer containing 0.1% Triton X-100. The wells are treated for 30 minutes at room temperature with aliquots (200 microliter) of a solution containing 10 mM sodium phosphate (pH 7.4), 0.5 M NaCl, 2% BSA, 0.1% Triton X-100 and 5mM EDTA (blocking solution).

After removing of the blocking solution from the wells, aliquots (50 microliter) of a solution containing horseradish peroxidase-labelled avidin (Vector Labs) is added to each well. The peroxidase-labelled avidin is diluted in PBS, 0.1% Triton X-100 according to the manufacturer's instructions.

The plates are incubated for 30 minutes at room temperature, then washed (4 x 200 microliter) with a solution of 10mM sodium phosphate (pH 7.4), 0.5 M sodium chloride 0.1% Triton X-100 and 5mM EDTA and then (1 x 200 microliter) with PBS containing 1mM EDTA.

The DNA-biotinylated probe-labelled avidin complexes are detected by adding to each well 150 microliter of a substrate reaction mixture containing 1.6 mg/ml o-phenylenediamine (Sigma) and 0.0125% hydrogen peroxide in 0.1 M sodium phosphate buffer adjusted to pH 6.0 by 0.05M citric acid solution. The plates are incubated for 30 minutes at room temperature in the dark and then the reaction is stopped by the addition of 4N sulfuric acid (50 microliter).

-37-

The contents of the plates were read in an Intermed Immuno Reader NJ-2000 spectrophotometric plate reader at wavelength 490 nm. The results are shown in Table 1:

5

Table 1

Absorbance (O.D. 490)

<u>[Amplified DNA]</u>	<u>Control</u> <sup>(a)</sup>
1 nmole	0.019
0.5 nmole	0.025
0.250 nmole	0.016
0.125 nmole	0.012

10

(a) Control means 100 picomoles of Bio-dUTP were fixed on the wells using ammonium acetate.

15

EXAMPLE 4

This example illustrates amplification using sticky-ended oligonucleotide complement pairs (E, E', F and F' as described). The desired sequence to be amplified is the same 48 base pair sequence as prepared in Example 1. In this experiment the incorporation of dATP[ $\alpha$ S] the gap-filling and the ligation step all take place at once.

20

1mM MgCl<sub>2</sub>, 20mM 2-mercaptoethanol, 100mg of bovine serum albumin per ml, 1.0 micromolar each of dATP[ $\alpha$ S], TTP[ $\alpha$ S], dATP and dTTP, and 100 picomoles of each of  $\alpha$ -<sup>32</sup>P-dATP and  $\alpha$ -<sup>32</sup>P-TTP (Specific activity 1000 cpm/picomole) are added to a 100 microliter buffer solution containing 50 mM Tris-HCl, pH 7.5.

25

To this solution are added a 5.0 microliter solution containing 100 picomoles of each of E and F' and each of phosphorylated F and E' and 0.1 picomole of 48 base pairs to be amplified. The resulting solution

30

-38-

is heated to 100°C for 3 minutes and allowed to cool to room temperature for 2 minutes, which allows the separation of the strands and hybridization of the set of two pairs to the target. Then 4 units of Klenow  
5 fragment of E. coli DNA polymerase-I and 2 units of T4-DNA ligase are added and the reaction incubated for 10 minutes at room temperature.

In order to cycle the process, the following steps are employed:

- 10 1. Heat for 3 minutes to 100°C.
2. Cool to room temperature for 2 minutes.
3. Add a mixture of 4 units of Klenow fragment of  
    E. coli DNA polymerase-I and 2 units of T4-DNA  
    ligase.
- 15 4. Incubate for 10 minutes at room temperature.

The cycle is repeated 22 times.

The resulting reaction mixture is loaded on a Sephadex G40/50 column (1x10cm) (Pharmacia) and eluted with  
double distilled water. Two distinct peaks are  
20 separated and monitored by a Geiger counter. The first peak appearing after elution of 5-7 ml of water, consists of the amplified 48 base pairs and the second peak appearing, after elution of 14-18 ml of water, consists of  $\alpha$ -<sup>32</sup>P-dATP and  $\alpha$ -<sup>32</sup>P-TTP.

25 The following examples illustrate amplification use oligonucleotide complement pairs attached to photosensitive molecules.



-39-

EXAMPLE 5

## PREPARATION OF N-(2-BROMOETHYL) PHTHALIMIDE

Potassium phthalimide (30.8 g, 0.17 M) was stirred at room temperature for 48 hours with 1,2-dibromoethane (63.9g, 0.34 M) and dry DMF (374 ml). (DMF is dimethyl formamide.) The precipitated KBr was filtered off, and the liquids were removed from the filtrate by distillation. The brown solid residue was recrystallized from cyclohexane-heptane (10:1) to give nearly colorless crystals (32.3 g, 72.3%, m.p. 73-75°C).

EXAMPLE 6

PREPARATION OF 8- HYDROXYPSORALEN (PREPARED BY THE PROCEDURE OF A. SCHONBERG ET AL., J. AM. CHEM. SOC., 72: 4826 (1950)).

A solution of 8-methylpsoralen (4.3 g, 19.90 mM) in dry benzene (100 ml) was added dropwise to magnesium iodide (from 10.16 g iodine and 0.97 g magnesium) in a mixture of dry ether (50 ml) and dry benzene (50 ml). The solvents were evaporated in vacuum at 120°C until the residue was practically dry and then further heated at 160-170°C for two hours.

The resulting solid residue was decomposed with dilute sulfuric acid and the filtered precipitate washed with water, suspended in dilute sodium bisulfite solution, filtered, washed with water and finally crystallized from dioxane as colorless crystals (m.p. 246°C).

-40-

EXAMPLE 7

## PREPARATION OF 1-(PSORALEN-8-YLOXY)-2-N-PHTHALIMIDOETHYL

To a stirred suspension of  $K_2CO_3$  (5 g, 35 mM) in dry acetone (100 ml), 8-hydroxypsoralen (1 g, 5 mM) and N-(-2-bromoethyl) phthalimide (1.77 g, 7 mM) were added. The mixture was refluxed (17 hours) and, after cooling, filtered. After removal of the solvent, the crude product was chromatographed twice on silica gel (Hexane-Diethyl ether 1:1) resulting in the product becoming a yellow oil (0.68 g, 39%).

NMR ( $DCl_3$ ) 3.31 (2H,t,J12Hz, $CH_2N$ ); 4.75 (2H,t,J12Hz, $CH_2O$ ), 6.38 (1H,d,J10Hz,3-H), 6.82 (1H,d,J2Hz,4-H), 7.40 (1H,s,5-H), 7.68 (1H,d,J2Hz,5-H), 7.75 (4HJ,d, PHTHAL), 7.8 (1H,d,J10Hz,4-H).

Mass spectrum, m/e 228, 229, 375 ( $m^+$ ).

Anal. Calcd. for  $C_{21}H_{13}NO_6$ : C,67.2; H,3.4; N,3.7.

Found: C,67.0; H,3.4; N,3.6.

EXAMPLE 8

## PREPARATION OF 2-(8-PSORALENOXY) ETHYLAMINE HYDROCHLORIDE

1-(psoralen-8-yloxy)-2-N-phthalimidoethyl (0.5 g, 1.3 mM), hydrazine hydrate (85% in water, 0.5 ml), and 95% ethanol (100 ml) were refluxed for 4 hours, followed by a second 0.5 ml addition of the hydrazine hydrate solution. After extending the reflux for 2 hours, no starting material remained, as determined by thin layer chromatography (diethyl ether). The ethanol was evaporated and the residue was taken up in 200 ml of 0.1N NaOH, followed by extraction

-41-

with three 50 ml portions of chloroform to give 135 mg (41.4%) of the crude amine. To prepare the hydrochloride, the amine was taken up in 100 ml of 1.2N HCl, which was extracted with three 30 ml portions of chloroform to remove impurities. Evaporation in vacuo of the acidic solution gave the crude hydrochloride, which was dissolved in enough absolute ethanol and precipitated by the addition of an equal volume of diethyl ether. After cooling overnight (4°C), 98 mg of pure product was collected:

NMR (CDCl<sub>3</sub>) as the amine 2.89 (2H,t,J9Hz,CH<sub>2</sub>N), 4.75 (2H,t,J9Hz,CH<sub>2</sub>O), 6.38 (1H,d,J10Hz,3-H), 6.82 (1H,d,J10Hz,4-H), 7.8 (1H, d, J10Hz, 4-H) 7.9 (2H,wide,S,NH<sub>2</sub>).

Mass spectrum m/e 201,245 (M<sup>+</sup>).

Anal. Calcd. for C<sub>13</sub>H<sub>12</sub>ClNO<sub>4</sub>: C,55.4; H,3.9.

Cl,12; N,4.9;

Found: C,55.1; H,3.9; Cl,12.4; N,4.8.

#### EXAMPLE 9

#### PREPARATION OF THE PHOTOACTIVABLE GROUPS ATTACHED TO OLIGONUCLEOTIDES

The desired sequence to be amplified was a fifty base pair sequence contained within a plasmid pBR322 containing 8 kilobases of HIV genome as doubly stranded DNA. The sequence has the nucleotide sequence as follows:

-42-

5' CACTT TGGAAAGGAC CAGCAAAGCT CCTCTGGAAA GGTGAAGGGG CAGTAG 4926-4976.  
3' GTGAA AACTTTCTG GTCGTTTCTGA GGAGACCTTT CCACTTCCCC GTCATC

A. Synthesis of phosphorylated oligonucleotides.

5 The following four oligodeoxyribonucleotides were prepared by the phosphoramidite solid support of Matteuci et al., J. Am. Chem. Soc., 103: 3185 (1981).

Two of them, I and J', were phosphorylated at 5' end, whereas J and I' were phosphorylated at 3' end. The sequences of I, I', J and J' are as follows:

10 I - 3'-GTGAAACCTTTTCCTGGTCGTTTCGAG-P-5'  
I' - 5'-CACTTTGGAAAGGACCAGCAAAGCTC-P-3'  
J - 3'-P-GAGACCTTTCCACTTCCCCGTCATC-5'  
J' - 5'-P-CTCTGGAAAGGTGAAGGGGCAGTAG-3'

15 In order to phosphorylate sequences I' and J at 3'-end, the following procedure was used.

Long chain alkylamine controlled pore glass (Pierce), pore diameter 500Å, particle size 125-177mm, 30 mg, was placed in an appropriate cell that fits to Applied Biosystems Inc. type 380B-02 DNA synthesizer.

20 The procedure included condensation of the phosphorylating agent (2-cyanoethoxy)-2-(2'-O-4,4'-dimethoxytrityloxyethylsufonyl)ethoxy-N,N-diisopropylamino-phosphine (Glen Corp. Inc.) followed by sequential condensation of  
25 diisopropylphosphoramidite (Applied Biosystems Inc.).

Automated Synthesis Procedures:

30 The procedure included condensation of the phosphorylating agent with the long chain alkylamine controlled pore glass using tetrazol as activating proton donor, and capping with acetic anhydride and dimethylaminopyridine in tetrahydrofuran and pyridine,

-43-

oxidation of the phosphite to the phosphate with  
I<sub>2</sub>/H<sub>2</sub>O/Lutidine/Tetrahydrofuran, following  
detritylation using 2% trichloroacetic acid in  
dichloromethane. Cycle time was approximately 30  
5 minutes.

The nucleotides of I' or J were sequentially  
condensed using the same cycle to the phosphorylating  
agent.

In order to synthesize 5'-phosphorylated  
10 oligonucleotides I and J', the cycle started with  
detritylation of the derivatized controlled pore glass  
with the appropriate nucleotides following the cycle  
steps as described above. In the last cycle, the  
phosphorylating agent was condensed. Yields at each  
15 step were essentially quantitative and were determined  
by collection and spectroscopic examination of the  
dimethoxytrityl alcohol released during detritylation.

#### Oligodeoxyribonucleotide Deprotection and Purification 20 Procedures:

The solid support was removed from the column  
and exposed to 1 ml concentrated ammonium hydroxide at  
60°C for 16 hours in a closed tube. Ammonia was  
removed and the residue was applied to a preparative  
25 12% polyacrylamide gel using a Tris-borate buffer (pH8)  
containing 7M urea. Electrophoresis was carried out at  
20 volts/cm for 5 hours after which the band containing  
the product was identified by UV shadowing of a  
fluorescent plate. The band was excised and eluted  
30 with 1 ml distilled water overnight at room  
temperature. This solution was filtered and the  
supernatant was extracted (3x300 microliter) with n-

-44-

butanol. The water phase was placed on a Sephadex G50 column (Pharmacia)(1x10cm) and eluted with double distilled water. The elution was monitored by UV absorbance at 260 nm and the appropriate fraction collected, quantitated by UV absorbance in a fixed volume and evaporated to dryness at room temperature in a vacuum centrifuge.

Kination of I' and J segments:

The oligodeoxyribonucleotides I' and J (5 nanomoles each) were lyophilized separately to dryness and redissolved in (50 microliter) of 50 mM of HEPES (Aldrich) pH 7.6, 10 mM  $MgCl_2$ , 10mM dithiotreitol and [ $\alpha^{32}P$ ]-ATP (10nM) specific activity =  $2.5 \times 10^3$  CPM/P mole.

Each tube was incubated with two units of T4-polynucleotide kinase for 40 minutes at 37°C for five minutes to inactivate the enzyme, and were loaded on sephadex columns G50 and eluted with water. The elutions were monitored by Geiger counter (Beckman) and lyophilized to dryness.

#### EXAMPLE 10

##### PREPARATION OF PHOTOACTIVABLE-OLIGODEOXYRIBONUCLEOTIDES

This example illustrates the labeling of any oligonucleotide with photoactivable group at 3' or 5' ends. In this example, x=y=any amino photoactivable group, such as 2-(8-psoralenoxy) ethylamine as described above in this invention or 5-aminomethyl-8-methoxypsoralen as described by J.B. Hansen et al., Tetrahedron Letters, 22: 1847-48 (1981), or 4'-aminomethyl-4-5; 8-trimethylpsoralen as described by Isaacs et al., Biochemistry, 16: 1058-64 (1977).

-45-

Phosphorylated oligodeoxyribonucleotides, 5'PI, 3'PJ, 3'PI' and 5'PJ' (10 nmoles) each were lyophilized separately to dryness in an Eppendorf tube. A solution of 0.2 M 1-Ethyl-3,3-dimethylaminopropylcarbodiimide (Sigma), (8-Psoralenoxy) ethylamine. Hydrochloride (10 mg) and 0.1 M of 1-methylimidazole buffer at pH 6.0 (1 ml) was made. 250 microliters from this solution was added to each of the Eppendorf tubes.

The mixtures were incubated for 24 hours at room temperature. The tubes were evaporated to dryness, the residues were redissolved in 30 microliters of 8% formamide and were purified by preparative electrophoresis on 12% polyacrylamide gels using a Tris-borate buffer pH 8 containing 7M urea. Electrophoresis was carried at 20 volts/cm for 3 hours after which the band containing the product was identified by U.V. shadowing of a fluorescent plate. Two bands were indentified that ran closely, the upper band showed a fluorescence upon exposure to long wave U.V. lamp.

The bands were excised and eluted with 1 ml distilled water overnight at room temperature. The mixtures were filtered and the supernatants were extracted (3 x 0.5 ml) with n-butanol. The aqueous phases were loaded on a Sephadex G50 column and eluted with water. The elution was monitored by U.V. absorbance at 260 nm and at 310 nm, and the appropriate fractions were collected, quantitated by U.V. absorbance in a fixed volume and evaporated to dryness at room temperature in a vacuum centrifuge. The yield of the photolabeling was 85%.

-46-

Radiolabeling of yI' and yJ at the 5' ends.

5 nanomoles each of lyophilized yI' and yJ were dissolved (50 microliters) in 5 nM of HEPES pH 7.6, 10mM MgCl<sub>2</sub>, 10 mM dithiothreitol and [<sup>32</sup>P]-ATP (10 nM, specific activity = 2.5 x 10<sup>3</sup> CPM/Pmole) and incubated with 1 unit of T4-polynucleotide kinase for 40 minutes at 37°C. The reaction mixtures were warmed to 80°C for 5 minutes to inactivate the enzyme. The content of each tube was loaded on a Sephadex G50 column (1 x 10 cm) and eluted with double distilled water.

The elution was monitored by Geiger counter and the appropriate fraction was collected, quantitated by radioactivity counting in a fixed volume and evaporated to dryness at room temperature in a vacuum centrifuge.

#### EXAMPLE 11

This example illustrates amplification of a 51 base pair sequence of HIV genome contained within the plasmid pBR322 using the photochemical process according to this embodiment of the invention.

The plasmid contains 8 kilobases of human immunodeficiency virus HIV genome sequence in which 51 base pairs were selected to be amplified from the POL region.

A total of 10 picomoles of the plasmid was linearized with EcoRI. One nanomole each of the following photoactivable-oligodeoxyribonucleotides were designed to produce a photoproduct adduct of 51 bases in length when I and J or I' and J' are joined together:



-47-

Ix = 3'-GTGAAACCTTTCCTGGTCGTTTCGAG-P-x-5'

I'y= 5'-\*PACTTTGGAAAGGACCAGCAAAGCTC-P-y-3'

Jy = 3'-y-P-GAGACCTTTCCTTCCCCGTCATCP\*-5'

J'x= 5'-x-P-CTCTGGAAAGGTGAAGGGGCAGTAG-3'

5 where x=y=2-(8-psoraleneoxy)ethylamino and p\* is the radioactive labeled phosphorus.

Sequences yI' and yJ were kinased at 5' end as described above. The radioactive labeled sequences are used for the analysis of the amplification efficiency when using the process and were dissolved in 100 ml of 1M guanine thiocyanate (GuSCN) solution.

10 The mixture was placed in a siliconized 1.5 ml polypropylene centrifuge tube (Sarstedt) and was flushed by bubbling the mixture with nitrogen for five minutes. The mixture was immersed in a 100°C water bath for two minutes, followed by quick cooling to 37°C and a five minute irradiation period. The cycle of heating, cooling and irradiating was repeated seven times.

20 The irradiation step was undertaken in an apparatus containing two 400-W General Electric mercury-vapor lamps (H400 A 33-1/T16), which were mounted on either side of a double-walled sample chamber, at a distance between centers of nine cm. The sample holder was maintained at 37°C by a thermostat and shielded by  $\text{Co}(\text{NO}_3)_2/\text{NaCl}/\text{H}_2\text{O}$  mixture (38:2:60 by weight) and with a 0.6 cm pyrex filter. The cobalt solution served as an ultraviolet filter, which allowed a maximum transmittance of 365 nm light and a window from approximately 340-380 nm. The intensity of the light at the surface of the inner sample was approximately  $100 \text{ mW/cm}^2$ .

-48-

After each irradiation, an aliquot of 5 ml was removed and kept for analysis.

After the seventh cycle, the aliquots were loaded on a 12% polyacrylamide gel using a Tris-borate buffer, pH8 containing 7M urea. Electrophoresis was carried out at 20 volt/cm for three hours. The gel was exposed to a film for four hours as presented in Fig. 2. Lane 1 shows the reaction mixture at time zero.

#### EXAMPLE 12

This example illustrates the coupling of T4-DNA ligase and Polymerase-I to polymeric supports.

##### I. Synthesis of the "linker arm".

1. O-(di-p-methoxytrityl)hexaethylene glycol. Hexaethylene glycol (Aldrich), (28.23 g, 100 mmole) is dissolved in dry pyridine (200 ml) and a solution of dimethoxytrityl chloride (16.95 g, 50 mmole) in dry pyridine (100 ml) is added dropwise under argon. The mixture is stirred at room temperature for 4 hours and the solvent is removed under reduced pressure (2 mm Hg). The oily yellow residue is extracted with ethyl acetate (300 ml) and washed with water (200 ml) and brine (2x200 ml). The organic layer is dried over anhydrous sodium sulfate and filtered. The solvent is removed and the residue is redissolved in dichloromethane (30 ml) which contain some pyridine (0.5 ml). This mixture is further purified by column chromatography on silica gel (5.5x45 cm) and eluted with a mixture of dichloromethane-methanol-pyridine (95:4:1: vol/vol). The product (23.3 g, yellow oil) has a  $R_F=0.42$  in the above solvents. The yield is (80%).

-49-

NMR = 7.46-7.20 (m, 9H, aromatic Hs), 6.80 (d, J=9.0 Hz, 4H, aromatic Hs), 3.77 (s, 6H, methoxy Hs), 3.66, 3.63 (2 s, 22H, CH<sub>2</sub>-CH<sub>2</sub>) 3.56 (t, J=5 Hz, 8H, CH<sub>2</sub>-OH), 3.21 (t, J=6.0 Hz, 2H, CH<sub>2</sub>-DMT), 2.49 (wide s, 1H, OH).  
5 Mass spectrum: m/e 584 (molecular ion), 553, 507, 477, 303 (DMT group).

2. O'-DMT-hexaethyleneglycol O-Methyl N,N-diisopropyl phosphoramidite.

O'-DMT-hexaethyleneglycol (1) (5.84 g,  
10 10 mmole) is dissolved in dry dichloromethane (30 ml) under argon. Dry N,N-di-isopropylethyl amine (5.16 g, 40 mmole) is added followed by dropwise addition of N,N-di-isopropyl O-methyl chlorophosphoramidite (Applied Biosystems) (1.97 g, 10 mmole). The solution  
15 is stirred at room temperature for 30 minutes, diluted with ethyl acetate (300 ml), washed with a saturated solution of sodium bicarbonate (100 ml) and with brine (100 ml). The organic layer is dried over anhydrous sodium sulfate and the solvent is removed under reduced  
20 pressure (15 mm Hg). Further drying is achieved by coevaporation with toluene (2x100 ml) and by lyophilization in dry benzene.

The product (7.21 g, yellow oil) has RF-0.63 in dichloromethane-methanol (95:5). The yield is (95%).  
25 NMR = 7.47-7.23 (m, 9H, aromatic Hs), 6.83 (d, J=9.0 Hz, 4H, aromatic Hs), 3.77 (s, 6H, methoxy), 3.77-3.59 (wide s, 22H, O-CH<sub>2</sub>CH<sub>2</sub>-O), 3.55 (d, J=12 Hz, 3H, CH<sub>3</sub>-O-P), 3.21 (t, J=6.0 Hz, 2H, CH<sub>2</sub>-O-DMT), 1.62 (d, 2H, CH<sub>3</sub>-CH), 1.24 (d, J=7Hz, 12H).

30 Mass spectrum 744 (molecular ion).

II. Attachment of the linker-arm to controlled pore glass.

Long chain alkyl amine-controlled pore glass (CPG), (Pierce), (500 A), (1 g), is placed in a sinter glass (medium) (50 ml), and washed with dry acetonitrile (3x10 ml) under argon. To the dried gel, a solution of 1H-Tetrazole (Aldrich), (0.5 g) in dry acetonitrile (12 ml), is added, followed by addition of a solution of O'-DMT-hexaethyleneglycol O-Methyl N,N-diisopropyl phosphoramidite (2 g) in dry acetonitrile (13 ml), under argon. The reaction mixture is agitated for 10 minutes followed by washings with tetrahydrofuran (3x25 ml). To the resulting gel, a capping solution containing acetic anhydride:tetrahydrofuran (1:1 vol/vol) (10 ml), and a solution of N,N-dimethylamino pyridine (0.5 g) in tetrahydrofuran (10 ml) are added, and the mixture is shaken for 6 minutes, followed by washings with tetrahydrofuran (3x20 ml). To the resulting solution an oxidation solution containing I<sub>2</sub> (0.1 Molar) in tetrahydrofuran : water : 2,6-lutidine (1:0.4:1), (10 ml) is added, and the mixture is shaken for 5 minutes, followed by washings with acetonitrile (3x20 ml). The measurement of the loading of the linker-arm on the controlled pore glass is done by adding a solution (1 ml) of para-toluenesulfonic acid in acetonitrile (0.1 Molar), to a 10 milligram of the derivative support, followed by measuring the absorbance at 498 nm. The loading is 185 micromoles of the linker-arm per 1 gram of the support. The dinethoxytrityl group is removed from the support, by a solution of trichloroacetic acid in dichloromethane

-51-

(3%). A new linker-arm could be condensed to the previous unprotected linker-arm in order to obtain a longer linker-arm, as desired.

5      III. Activation of the CPG-linker-arm with tresyl chloride.

10      The dried support-linker-arm (1 g), is placed in a sinter glass as above, and washed with dry acetone (3x50 ml), followed by addition of dry acetone (5 ml) and 260 microliters of dry pyridine. Tresyl chloride (Fluka), (180 microliters) is added to the suspension dropwise for 1 minute, under shaking. After reaction for 15 minutes at 0°C, the gel is washed twice with (20 ml) of the following, 30:70, 50:50, and 70:30 of 5 mM HCL:acetone (vol/vol) and finally with water, 15      50:50 water:acetone and acetone, (20 ml each) dried, and stored in desiccator until required.

IV. Coupling of T4-DNA Ligase to tresylated CPG-linker-arm.

20      40 units of T4-DNA ligase (BRL), are dissolved in 0.1M  $\text{NaH}_2\text{PO}_4$ , pH 7.5, containing 1 mM EDTA, 50 micromolar ATP, 10% glycerol to a final volume of 200 microliters and kept at 40°C. 100 milligrams of dry tresylated CPG-linker-arm is transferred to the enzyme solution. The resulting gel slurry is mixed 25      "end over end" at 4C for 16 hours. Residual reactive tresyl groups on the linker-arm are removed by adding 0.1M dithiotreitol, (2 hours, 4°C). After coupling, the gel is washed free of uncoupled enzyme by washing four times in 0.1 M  $\text{NaH}_2\text{PO}_4$ , pH 7.5, 1 mM EDTA, 10 mM 30      dithiothreitol (DTT), 0.5 M NaCl followed by two washes in 0.1 M NaAc, pH 5.0. Finally, the gel is

-52-

equilibrated in 66 mM Tris-HCl, pH 7.6, 6.6 mM MgCl<sub>2</sub>, 10 mM DTT, 10% glycerol and stored at 4°C.

V. Coupling of Klenow fragment of E. coli DNA Polymerase-I to tresylated CPG-linker-arm.

5                   40 units of Klenow fragment of E. coli DNA Polymerase-I (Bethesda Research Labs.) are dissolved in 200 microliters of buffer consisting of 1.5 mM ATP; 30 mM Tris acetate buffer at pH 7.9; 60 mM sodium acetate; 10 mM magnesium acetate; and added to a  
10                   lyophilized mixture of 80 microgram Poly (dA)-20 microgram oligo (dT). To this mixture is added 100 milligrams of dry tresylated CPG-linker-arm. The resulting gel slurry is mixed "end-over-end" at 4°C for 10 hours. Residual reactive tresyl groups on the  
15                   linker-arm are removed by adding 0.1 mM dithiothreitol (DTT), (2 hours, 4C). After coupling, the gel is washed free of uncoupled enzyme by washing (4x0.5 ml) in a buffer consisting of 60 mM sodium acetate, 30 mM Tris acetate, 10 mM magnesium acetate at pH 8.0 and  
20                   0.25 mM dithiotreitol. Finally, the gel is equilibrated in 66 mM Tris-HCl pH 7.6; 6.6 mM magnesium chloride; 10 mM DTT; 10% glycerol and stored at 4°C.

25                   The following examples illustrate the use of these immobilized enzymes in the method of the application.

-53-

EXAMPLE 13

Step 1: The synthesis of the Chlamydia sequence.

5 The desired sequence to be amplified using the gap-filling/ligating system of joining the oligonucleotides is a 88 base pair that coded for Chlamydia trachomatis cryptic plasmid. This particular sequence is a four times repeat sequence of 22 base pairs, that has been obtained by the sequencing of the 10 6.5 kilobase plasmid derived from Chlamydia trachomatis (serotype L) inserted into the unique Bam H1/ Sst1 sites of the PUC18 plasmid. The 6.5 Kb are sequenced using the dideoxy chain termination method of Sanger et al. P.N.A.S. 83, 1911 (1986).

15 The 22 repeated base pair has the following sequence:

5'-(GTCTACCAACCAAGAGTTGCAA)x4-3'

3'-(CAGATGGTGGTTCTCAACGTTT)x4-5'

20 The construction of the two pairs is as follows:

Y = 5'-AGTCTACCAACCAAGAGTTGC-3'

Z = 3'- CAGATGGTGGTTCTCAACGT-5'

Y represents in this case oligonucleotides A and B in the oligonucleotide complement pairs.

25 Z represents in this case oligonucleotides A' and B' in the oligonucleotide complement pairs. When hybridized to the target sequence, Y and Z form a gap of two complementary base pairs, A-T.

30 The two oligodeoxyribonucleotides described above are synthesized and purified by the following procedure.

-54-

I. Automated Synthesis Procedures. (according to Matteucci et al., J. Am. Chem. Soc., 103, 31-5, (1981).

The 2-cyanoethyl phosphoramidites are purchased from Applied Biosystems Inc. The procedure includes condensation of nucleoside phosphoramidites to a nucleoside-derivatized controlled pore glass bead support, (500A) 30 mg, using NDA synthesizer from Applied Biosystems Inc., Type 380B-02. The cycles includes detritylation with 2% trichloroacetic acid in dichloromethane; condensation using tetrazole as an activating proton donor; capping with acetic anhydride and dimethylaminopyridine; oxidation of the phosphite to the phosphate with 0.1 M I<sub>2</sub>/H<sub>2</sub>O/Lutidine/Tetrahydrofuran, following detritylation using 2% trichloroacetic acid in dichloromethane. Cycle time is approximately 30 minutes. Yields at each step are essentially quantitative and are determined by collection and spectroscopic examination of the dimethoxytrityl alcohol released during detritylation.

II. Oligodeoxyribonucleotide Deprotection and Purification Procedures.

The solid support is removed from the column and exposed to 1 ml concentrated ammonium hydroxide at 60° C for 16 hours in a closed tube. Ammonia is removed and the residue is applied to a preparative 12% polyacrylamide gel using a Tris-borate buffer (pH 8) containing 7M urea. Electrophoresis is carried out at 20 volts/cm for 5 hours after which the band containing the product is identified by UV shadowing of a fluorescent plate. The band is excised and eluted with 1 ml double distilled water overnight at room temperature. This solution is filtered and the



-55-

supernatant is extracted (3x300 microliter) with n-butanol. The water phase is placed on a Sephadex G-50 column (Pharmacia) (1x10 cm). The elutions are monitored by UV absorbance at 260 nm and the appropriate fraction collected, quantitated by UV absorbance in a fixed volume and evaporated to dryness at room temperature in a vacuum centrifuge.

III. 5'-Phosphorylation of A, B, A' and B'.

The oligodeoxyribonucleotides Y and Z (5 nanomoles each) are lyophilized separately to dryness and redissolved in (50 microliter) of 50 mM HEPES (Aldrich) pH 7.6, 10 mM,  $MgCl_2$ , 10 mM dithiotreitol and [ $^{32}P$ ]-ATP (10nM). Specific activity =  $2.5 \times 10^3$  cpm/pmole. Each tube is incubated with two units of  $T_4$ -polynucleotide kinase for 40 minutes at 37°C for five minutes to inactivate the enzyme, and are loaded on Sephadex columns G50 and eluted with water. The elutions are monitored by gamma counter (Beckman) and lyophilized to dryness.

#### EXAMPLE 14

##### I. Purification of Chlamydial DNA

Chlamydia trachomatis LGV2 strain is grown in McCoy cells in the presence of 1 microgram cycloheximide/ml until about 1.0 IFU (inclusion-forming unit) per cell. The cells are grown for 3 days at which time 80-90% of them showed visible inclusions and are harvested and stored at -70°C until tested. Control microbial strains are obtained from routine isolations (Department of Microbiology, Ben-Gurion University) and identified by standard procedures

-56-

(Lennette et al., Manual of Clinical Microbiology, 3rd edition, Washington (1980)). The strains include isolates of E. coli and Streptococcus agalactiae.

5 The microbial strains are grown on plates or in liquid medium. For the test, a suspension of each of the microbes is boiled at an alkaline pH (0.3 M NaOH), chilled on ice and neutralized with HCl.

10 The Chlamydial cell culture harvests of 100 ml are disrupted with a glass-Teflon homogenizer and centrifuged at 1.400 x g for 5 minutes to remove cell debris. The supernatant is layered onto 30% (vol/vol) Renografin -76 (E.R. Squibb, Princeton U.S.A.) diluted with 20 mM Tris-150 mM HCl (THCl) (pH 7.5); Renografin -76, (a solution of diatrizoate meglumine and diatrizoate sodium in citrate buffer).  
15 After centrifugation at 50,000 x g for 45 minutes, the pellets are resuspended in 1.0 ml of THCl, layered onto 30 to 60% (vol/vol), Renografin -76 gradients in THCl, and centrifuged at 50.000 x g for 2 hr.  
20 Two diffuse bands are visible in the middle region of the gradient; these fractions are collected, diluted with THCl and centrifuged at 50.000 x g for 45 min. The pellets are resuspended in 0.5 ml of THCl.

## II. DNA Extraction

25 The following procedure is based on the method described by (Wenman et al., Nature 296:68-70 (1982)).

30 The Chlamydial suspension is homogenized before the addition of 50 mM Tris containing 20 mM Na<sub>2</sub> EDTA 20% (wt/vol) sucrose, 0.7% (wt/vol) N-lauroylsarcosine; and 200 microgram of proteinase K. This mixture is incubated at 55°C for 15 min. and then

-57-

at 37°C for 45 min. An equal volume of phenol-chloroform is added and the mixture is vortexed and centrifuged at 2,000 x g for 10 min. Nucleic acids are precipitated from the aqueous phase by addition of a  
5 1/20 volume of 5M NaCl and 2 volumes of ethanol. After two further cycles of centrifugation and precipitation, the DNA is measured spectrophotometrically, precipitated once more, and redissolved in 10 mM Tris with 1 mM EDTA (pH 7.0) at a concentration of 200  
10 microgram/ml.

DNA is digested in 4 to 10 microgram amounts at a concentration of 80 microgram/ml with 4 units of BAM-HI (B1) per microgram of DNA for 2 to 3 hours, under the conditions recommended by the manufacturer.

15

#### EXAMPLE 15

This example illustrates the gap filling-ligation amplification steps using immobilized enzymes employing the apparatus as shown in Figure 3.

20

A mixture of polymeric beads 1, (10 mg beads) of CPG-T<sub>4</sub>-DNA ligase from Example 12. IV and (10 mg beads) of CPG-Klenow fragment of E. coli. DNA Polymerase-I from Example 12.V, are placed in plastic cylinder 2 (inner volume ~ 0.5 ml), where the edges of cylinder 2 contain plastic covers 3a, 3b, having  
25 inlet 4 and outlet 5 that connect to plastic tubing 6, 7. The bottom of cylinder 2 is filled with siliconized glass wool 8, which holds beads 1.

25

30

Inlet 4 and outlet 5 are attached to plastic tubing 6, 7, the tubing has a diameter of 0.5 mm. Plastic tubing 7 is attached to stainless steel coil 9, which is immersed in hot oil bath 10, containing

-58-

silicon oil at 90°C. Stainless steel coil 9 is attached by tube 11 to second stainless coil 12. Coil 12 is immersed in a water bath 13, held at room temperature. Coils 9 and 12 are also 0.5 mm in diameter. Coil 12 is attached to plastic tube 14, having a diameter of 0.5 mm. Tube 14 is connected to peristaltic pump 15 and thereafter to cell 2 through inlet 4. The pump pushes the solution through the system at a rate of two drops per minute.

Step 1. Protection of Y and Z Oligonucleotides from the 3'-5' Exonuclease Activity of DNA Polymerase I and to Form Blunt Ends.

100 picomole of each 5'-phosphorylated Y and Z are mixed together and dissolved in 1 millimeter of 1 mM  $MgCl_2$ , 20 mM 2-mercaptoethanol, 100 mg of bovine serum albumin per ml and 50 mM Tris-HCl (pH 7.5), and 0.1 mM of dATP[ $\alpha S$ ] and TTP[ $\alpha S$ ](Sigma).

The resulting solution is heated to 100°C for 2 minutes which allows separation of the strands and hybridization of the Y and Z to the target. Then 5 units of Klenow fragment of *E. coli* DNA polymerase-I are added and the reaction is incubated for 10 minutes at room temperature. The pairs are now blunt-ended and protected against exonuclease activity of polymerase.

Step 2. 1 microgram of Bam HI digested DNA from Example 14.II above is dissolved in the buffer resulted from step 1. To this buffer is added 1 nanomole of dATP and TTP and 100 p moles of each of  $\alpha$ - $^{32}P$ [dATP] and  $\alpha$ - $^{32}P$ [TTP]. Specific activity 1000 cpm/picamole.

This solution is injected through inlet 4 of the apparatus and the cycling is started by operating peristaltic pump 15.

-59-

After 3 hours of cycling the solution through cell 2 containing the immobilized enzymes, the solution is loaded on a Sephadex column G-40/50 (1 x 10 cm) and eluted with doubly distilled water. Fractions of 1 ml are collected and monitored by a Geiger counter.

Two distinct bands are detected, the first is eluted in a pool of 4-7 ml containing labeled amplified joined oligonucleotide products by gap filling/ligation process. This pool has about 115000 cpm whereas the second pool that eluted in 14-17 ml has 80,000 cpm.

-60-

WHAT IS CLAIMED IS:

1. A process for detecting the presence or absence of at least one specific nucleic acid target sequence, or a portion thereof, by amplifying the nucleic acid target sequence(s), or portion(s) thereof, in a sample containing a nucleic acid or a mixture of nucleic acids, or distinguishing between at least two different nucleic acid sequences in the sample, wherein the sample is suspected of containing the target sequence(s), comprising the steps of:

(a) treating the sample with at least two oligonucleotides for each strand of a target sequence, under hybridizing conditions, wherein the oligonucleotides are selected so as to be sufficiently complementary to each strand of the target sequence(s) or portion(s) thereof to hybridize therewith;

(b) joining the oligonucleotides, hybridized after step (a) to a strand of the target sequence(s), or portion(s) thereof, to each other, thereby forming a joined oligonucleotide product;

(c) treating the hybridized oligonucleotides of step (b) under denaturing conditions to separate the joined oligonucleotide product(s) from the target sequence(s), or portion(s) thereof, if the target sequence(s), or portion(s) thereof, are present in the sample and to produce single-stranded molecules;

(d) treating the single-stranded molecules produced in step (c) with at least two oligonucleotide complement pairs, under hybridizing conditions, wherein each oligonucleotide complement pair comprises two oligonucleotides selected so as to be sufficiently

-61-

complementary to each other, to each strand of the target sequence(s), or portion(s) thereof, and to the joined oligonucleotide product(s) to hybridize therewith;

35           (e) joining the oligonucleotides, hybridized to the strands of the target sequence(s), or portion(s) thereof, and hybridized to the joined oligonucleotide product(s) formed in step (b), to each other, thereby forming additional joined oligonucleotide product(s),  
40           resulting in the amplification of the target sequence(s), or portion(s) thereof; and

          (f) detecting the joined oligonucleotide product(s).

2. The process of claim 1, further including step

5           (e') treating the hybridized oligonucleotides of step (e) under denaturing conditions to separate the hybridized oligonucleotides and produce single-stranded molecules, wherein steps (d), (e) and (e') are repeated a desired number of times.

3. The process of claim 1, wherein the nucleic acid target sequence(s), or portion(s) thereof, is double-stranded DNA and its strands are separated before or during step (a).

4. The process of claim 1, wherein the nucleic acid target sequence(s), or portion(s) thereof, is single-stranded DNA or RNA.

-62-

5. The process of claim 1, wherein the oligonucleotides are oligodeoxyribonucleotides.

6. The process of claim 1, wherein the oligonucleotide complement pairs are present as a molar excess in the range of  $10^5$  to  $10^{15}$  pairs per nucleic acid target sequence(s), or portion(s) thereof.

7. The process of claim 1, wherein a gap of one or more bases is present between the oligonucleotides when the oligonucleotides are hybridized to the target sequence(s), or portion(s) thereof, or to the joined oligonucleotide product(s).

8. The process of claim 7, wherein the gap is filled by catalytic agents.

9. The process of claim 8, wherein the catalytic agents are a combination comprising a DNA polymerase and a DNA ligase.

10. The process of claim 8, wherein the catalytic agents are immobilized on polymeric supports.

11. The process of claim 10, wherein the DNA polymerase is selected from the group consisting of E. coli DNA polymerase-I, Klenow fragments of E. coli DNA polymerase-I, T4 DNA polymerase, reverse transcriptase and the ligase is selected from the group consisting of E. coli DNA ligase or T4 DNA ligase.



-63-

12. The process of claim 10, wherein the polymerase and ligase are heat stable.

13. The process of claim 12, wherein the heat stable polymerase and ligase are isolated from a thermophilic bacteria.

14. The process of claim 7, wherein the oligonucleotides are selected so that the gap formed when the oligonucleotides are hybridized to the target sequence(s), or portion(s) thereof, or joined  
5 oligonucleotide product(s) can be filled by no more than two complementary nucleotides.

15. The process of claim 7, wherein the oligonucleotides are selected so that the gap formed when the oligonucleotides are hybridized to the target sequence(s), or portion(s) thereof, or to the joined  
5 oligonucleotide product(s), can be filled by no more than two complementary nucleotides and one other nucleotide, the other nucleotide being non-complementary to either of the two complementary nucleotides.

16. The process of claim 8, wherein deoxyribonucleotide triphosphates are added to the sample mixture in steps (a) and (d).

17. The process of claim 16, wherein the oligonucleotides and/or deoxyribonucleic triphosphates are modified to be resistant to 3'-->5' exonuclease activity.

-64-

18. A process for detecting the presence or absence of at least one specific nucleic acid target sequence, or a portion thereof, by amplifying the nucleic acid target sequence(s), or portion(s) thereof, in a sample containing a nucleic acid or a mixture of nucleic acids, or distinguishing between at least two different nucleic acid sequences in the sample, wherein the sample is suspected of containing the target sequence(s), comprising:

(a) treating the sample with at least two oligonucleotides for each strand of a target sequence, under hybridizing conditions, wherein the oligonucleotides are selected so as to be sufficiently complementary to each strand of the target sequence(s), or portion(s) thereof, to hybridize therewith and are further selected so a gap of one or more bases is present between the oligonucleotides when the oligonucleotides are hybridized to the target sequence(s), or portion(s) thereof;

(b) joining the oligonucleotides, hybridized to a strand of the target sequence(s), or portion(s) thereof, to each other, thereby forming a joined oligonucleotide product;

(c) treating the hybridized oligonucleotides of step (b) under denaturing conditions to separate the joined oligonucleotide product(s) from the target sequence(s), or portion(s) thereof, if the target sequence(s) are present in the sample and to produce single-stranded molecules;

(d) treating the single-stranded molecules produced in step (c) with at least two oligonucleotide complement pairs, under hybridization conditions,

-65-

wherein each oligonucleotide complement pair comprises two oligonucleotides selected so as to be sufficiently complementary to each other, to each strand of the target sequence(s), or portion(s) thereof, and to the joined oligonucleotide product(s) to hybridize therewith and further selected so a gap of one or more bases is present between the oligonucleotides when the oligonucleotides are hybridized to the target sequence(s), or portion(s) thereof, or to the joined oligonucleotide product(s);

(e) joining the oligonucleotides, hybridized to the strands of the target sequence(s), or portion(s) thereof, and to the joined oligonucleotide product(s) formed in step (b), to each other, thereby forming additional joined oligonucleotide product(s), resulting in the amplification of the target sequence(s), or portion(s) thereof; and

(f) detecting the joined oligonucleotide product(s).

19. The process of claim 18, further including step

(e') treating the hybridized oligonucleotides of step (e) under denaturing conditions, to separate the hybridized oligonucleotides and to produce single-stranded molecules, wherein steps (d), (e) and (e') are repeated a desired number of times.

20. The process of claim 18, wherein the gap is filled by catalytic agents.

-66-

21. The process of claim 20, wherein the catalytic agents are a combination comprising a DNA polymerase and a DNA ligase.

22. The process of claim 20, wherein the catalytic agents are immobilized on polymeric supports.

23. The process of claim 21, wherein the DNA polymerase is selected from the group consisting of E. coli DNA polymerase-I, Klenow fragments of E. coli DNA polymerase-I, T4 DNA polymerase, reverse transcriptase and the ligase is selected from the group consisting of E. coli DNA ligase or T4 DNA ligase.

24. The process of claim 21, wherein the polymerase and ligase are heat stable.

25. The process of claim 24, wherein the heat stable polymerase and ligase are isolated from a thermophilic bacteria.

26. The process of claim 18, wherein the oligonucleotides are selected so that the gap, formed when the oligonucleotides are hybridized to the target sequence(s), or portion(s) thereof, or to joined oligonucleotide product(s), can be filled by no more than two complementary nucleotides.

27. The process of claim 18, wherein the oligonucleotides are selected so that the gap, formed when the oligonucleotides are hybridized to the target sequence(s), or portion(s) thereof, or joined

-67-

5 oligonucleotide product(s), can be filled by no more than two complementary nucleotides and one other nucleotide, the other nucleotide being non-complementary to either of the two complementary nucleotides.

28. A process for detecting the presence or absence of at least one specific nucleic acid target sequence, or a portion thereof, by amplifying the nucleic acid target sequence(s), or portion(s) thereof, in a sample containing a nucleic acid or a mixture of nucleic acids, or distinguishing between at least two different nucleic acid sequences in the sample, wherein the sample is suspected of containing the target sequence(s), comprising the steps of:

10 (a) treating the sample with a least two oligonucleotides for each strand of a target sequence, under hybridizing conditions, wherein the oligonucleotides are selected so as to be sufficiently complementary to each strand of the target sequence(s), or portion(s) thereof, to hybridize therewith, wherein the oligonucleotides are attached to a photoactivatable group at at least one end of the oligonucleotide, and wherein the photoactivatable group at the end of one oligonucleotide is adjacent to the photoactivatable group at the end of another oligonucleotide when both of the oligonucleotides are hybridized to a target sequence(s), or portion(s) thereof;

25 (b) joining the oligonucleotides, hybridized after step (a) to a strand of the target sequence(s), or portion(s) thereof, to each other by activating the photoactivatable groups to form a bond with adjacent

-68-

photoactivatable groups, thereby forming a joined oligonucleotide product;

30 (c) treating the sample under denaturing conditions to separate the joined oligonucleotide product(s) from the target sequence(s), or portion(s) thereof, if the target sequence(s), or portion(s) thereof, are present in the sample;

35 (d) treating the sample with at least two oligonucleotide complement pairs, wherein each oligonucleotide complement pair comprises two oligonucleotides selected so as to be sufficiently complementary to each other, to each strand of the target sequence(s), or portion(s) thereof, and to the  
40 joined oligonucleotide product(s) to hybridize therewith and wherein each oligonucleotide is attached to a photoactivatable group at at least one the end of the oligonucleotide and wherein the photoactivatable group at the end of one oligonucleotide is adjacent to  
45 the photoactivatable group at the end of another oligonucleotide when both of the oligonucleotides are hybridized to a target sequence(s), or portion(s) thereof;

50 (e) joining the oligonucleotides, hybridized to the strands of the target sequence(s), or portion(s) thereof, and hybridized to the joined oligonucleotide product(s) formed in step (b), to each other by activating the photoactivatable groups to form a bond with adjacent photoactivatable groups, thereby forming  
55 additional joined oligonucleotide product(s), resulting in the amplification of the target sequence(s), or portion(s) thereof; and

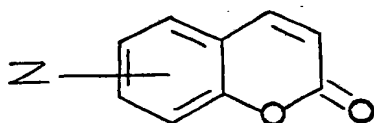
-69-

(f) detecting the joined, oligonucleotide product(s).

29. The process of claim 28, further including step (e') treating the hybridized oligonucleotides of step (e) under denaturing conditions to separate the hybridized oligonucleotides and to produce single-stranded molecules, wherein steps (d), (e) and (e') are repeated a desired number of times.

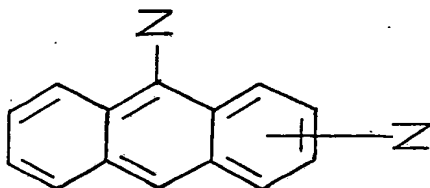
30. The process of claim 28, wherein the oligonucleotide complement pairs are present as a molar excess in the range of  $10^5$  to  $10^{15}$  pairs per nucleic acid target sequence(s), or portion(s) thereof.

31. The process of claim 28, wherein the photoactivatable groups are selected from the group consisting of psoralens,



Coumarin derivatives

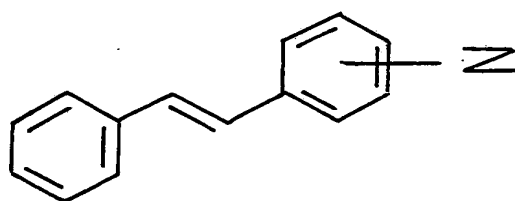
where Z is H, methoxy, acetoxy,  $C_1$ - $C_5$  alkyl, halogen and di- or tri- derivatives of these groups;



Anthracene derivatives

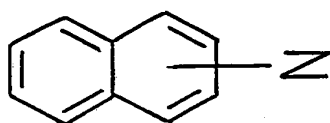
where Z is H, CN, alkyl or halogen;

-70-



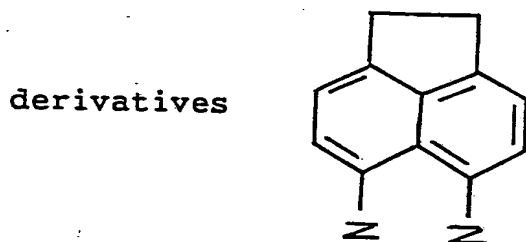
Stilbene derivatives

where Z is H, CN, alkyl, halogen, CO<sub>2</sub>H, or alkyl ester;



Naphthalene derivatives

where Z is H, CN, -O-Alkyl, halogen or carboxy; and



Acenaphthylene

derivatives

where Z is H, CN, alkyl, halogen or carboxy.

32. A process for amplifying at least one specific nucleic acid target sequence, or a portion thereof, in a sample containing a nucleic acid or a mixture of nucleic acids, comprising the steps of:

5

(a) treating the sample with at least two oligonucleotides for each strand of a target sequence, under hybridizing conditions, wherein the oligonucleotides are selected so as to be sufficiently complementary to each strand of the target sequence(s), or portion(s) thereof, to hybridize therewith;

10

(b) joining the oligonucleotides, hybridized after step (a) to a strand of the target sequence(s), or portion(s) thereof, to each other, thereby forming a joined oligonucleotide products;



-71-

15 (c) treating the hybridized oligonucleotides  
of step (b) under denaturing conditions to separate the  
joined oligonucleotide product(s) from the target  
sequence(s), or portion(s) thereof, if the target  
sequence(s), or portion(s) thereof, are present in the  
20 sample and to produce single-stranded molecules;

(d) treating the single-stranded molecules  
produced in step (c) with at least two oligonucleotide  
complement pairs, wherein each oligonucleotide  
complement pair comprises two oligonucleotides selected  
25 so as to be sufficiently complementary to each other,  
to each strand of the target sequence(s), or portion(s)  
thereof, and to the joined oligonucleotide product(s)  
to hybridize therewith; and

(e) joining the oligonucleotides, hybridized  
30 to the strands of the target sequence(s), or portion(s)  
thereof, and hybridized to the joined oligonucleotide  
product(s) formed in step (b), to each other, thereby  
forming additional joined oligonucleotide product(s),  
resulting in the amplification of the target  
35 sequence(s), or portion(s) thereof.

33. The process of claim 32, further  
including step

(e') treating the hybridized oligonucleotides  
of step (e) under denaturing conditions to separate the  
5 hybridized oligonucleotides and produce single-stranded  
molecules, wherein steps (d), (e) and (e') are repeated  
a desired number of times.

34. The process of claim 32, wherein the  
nucleic acid target sequence(s), or portion(s) thereof,

-72-

is double-stranded DNA and its strands are separated before or during step (a).

35. The process of claim 32, wherein the nucleic acid target sequence(s), or portion(s) thereof, is single-stranded DNA or RNA.

36. The process of claim 32, wherein the oligonucleotides are oligodeoxyribonucleotides.

37. The process of claim 32, wherein the oligonucleotide complement pairs are present as a molar excess in the range of  $10^5$  to  $10^{15}$  pairs per nucleic acid target sequence(s), or portion(s) thereof.

38. The process of claim 32, wherein a gap of one or more bases is present between the oligonucleotides when the oligonucleotides are hybridized to the target sequence(s), or portion(s) thereof, or to the joined, oligonucleotide product(s).

39. The process of claim 38, wherein the gap is filled by catalytic agents.

40. The process of claim 39, wherein the catalytic agents are a combination comprising a DNA polymerase and a DNA ligase.

41. The process of claim 39, wherein the catalytic agents are immobilized on polymeric supports.

-73-

42. The process of claim 40, wherein the DNA polymerase is selected from the group consisting of E. coli DNA polymerase-I, Klenow fragments of E. coli DNA polymerase-I, T4 DNA polymerase, reverse transcriptase and the ligase is selected from the group consisting of E. coli DNA ligase or T4 DNA ligase.

43. The process of claim 40, wherein the polymerase and ligase are heat stable.

44. The process of claim 43, wherein the heat stable polymerase and ligase are isolated from a thermophilic bacteria.

45. The process of claim 38, wherein the oligonucleotides are selected so that the gap formed when the oligonucleotides are hybridized to the target sequence(s), or portion(s) thereof, or joined oligonucleotide product(s) can be filled by no more than two complementary nucleotides.

46. The process of claim 38, wherein the oligonucleotides are selected so that the gap formed when the oligonucleotides are hybridized to the target sequence(s), or portion(s) thereof, or to the joined oligonucleotide product(s), can be filled by no more than two complementary nucleotides and one other nucleotide, the other nucleotides being non-complementary to either of the two complementary nucleotide.

-74-

47. The process of claim 40, wherein deoxyribonucleotide triphosphates are added to the sample mixture in steps (a) and (d).

48. The process of claim 47, wherein the oligonucleotides and/or deoxyribonucleic triphosphates are modified to be resistant to 3'-->5' exonuclease activity.

49. A process for amplifying at least one specific nucleic acid target sequence, or a portion thereof, in a sample containing a nucleic acid or a mixture of nucleic acids, comprising the steps of:

5 (a) treating the sample with at least two oligonucleotides for each strand of a target sequence, under hybridizing conditions, wherein the oligonucleotides are selected so as to be sufficiently complementary to each strand of the target sequence(s),  
10 or portion(s) thereof, to hybridize therewith and are further selected so a gap of one or more bases is present between the oligonucleotides when the oligonucleotides are hybridized to the target sequence(s), or portion(s) thereof;

15 (b) joining the oligonucleotides, hybridized to a strand of the target sequence(s), or portion(s) thereof, to each other, thereby forming a joined oligonucleotide product;

20 (c) treating the hybridized oligonucleotides of step (b) under denaturing conditions to separate the joined oligonucleotide product(s) from the target sequence(s), or portion(s) thereof, if the target sequence(s) are present in the sample and to produce single-stranded molecules;

-75-

25 (d) treating the single-stranded molecules  
produced in step (c) with at least two oligonucleotide  
complement pairs, wherein each oligonucleotide  
complement pair comprises two oligonucleotides selected  
so as to be sufficiently complementary to each other,  
30 to each strand of the target sequence(s), or portion(s)  
thereof, and to the joined oligonucleotide product(s)  
to hybridize therewith and further selected so a gap of  
one or more bases is present between the  
oligonucleotides when the oligonucleotides are  
35 hybridized to the target sequence(s), or portion(s)  
thereof, or to the joined oligonucleotide product(s);  
and

(e) joining the oligonucleotides, hybridized  
to the strands of the target sequence(s), or portion(s)  
40 thereof, and to the joined, oligonucleotide product(s)  
formed in step (b), to each other, thereby forming  
additional joined oligonucleotide product(s), resulting  
in the amplification of the target sequence(s), or  
portion(s) thereof.

50. The process of claim 49, further  
including step

(e') treating the hybridized oligonucleotides  
of step (e) under denaturing conditions, to separate  
5 the hybridized oligonucleotides and to produce single-  
stranded molecules, wherein steps (d), (e) and (e') are  
repeated a desired number of times.

51. The process of claim 49, wherein the gap  
is filled by catalytic agents.

-76-

52. The process of claim 51, wherein the catalytic agents are a combination comprising a DNA polymerase and a DNA ligase.

53. The process of claim 51, wherein the catalytic agents are immobilized on polymeric supports.

54. The process of claim 52, wherein the DNA polymerase is selected from the group consisting of E. coli DNA polymerase-I, Klenow fragments of E. coli DNA polymerase-I, T4 DNA polymerase, reverse transcriptase and the ligase is selected from the group consisting of E. coli DNA ligase or T4 DNA ligase.

55. The process of claim 52, wherein the polymerase and ligase are heat stable.

56. The process of claim 55, wherein the heat stable polymerase and ligase are isolated from a thermophilic bacteria.

57. The process of claim 49, wherein the oligonucleotides are selected so that the gap, formed when the oligonucleotides are hybridized to the target sequence(s), or portion(s) thereof, or to joined oligonucleotide product(s), can be filled by no more than two complementary nucleotides.

58. The process of claim 49, wherein the oligonucleotides are selected so that the gap, formed when the oligonucleotides are hybridized to the target sequence(s), or portion(s) thereof, or joined

-77-

5 oligonucleotide product(s), can be filled by no more  
than two complementary nucleotides and one other  
nucleotide, the other nucleotide being non-  
complementary to either of the two complementary  
nucleotides.

59. A process for amplifying at least one  
specific nucleic acid target sequence, or a portion  
thereof, in a sample containing a nucleic acid or a  
mixture of nucleic acids, comprising the steps of:

5 (a) treating the sample with a least two  
oligonucleotides for each strand of a target sequence,  
under hybridizing conditions, wherein the  
oligonucleotides are selected so as to be sufficiently  
complementary to each strand of the target sequence(s),  
10 or portion(s) thereof, to hybridize therewith and  
wherein each oligonucleotide is attached to a  
photoactivatable group at the end of the  
oligonucleotide that is adjacent to another  
oligonucleotide when both of the oligonucleotides are  
15 hybridized to a target sequence(s), or portion(s)  
thereof.

(b) joining the oligonucleotides, hybridized  
after step (a) to a strand of the target sequence(s),  
or portion(s) thereof, to each other by activating the  
20 photoactivatable groups to form a bond with adjacent  
photoactivatable groups, thereby forming a joined  
oligonucleotide product;

(c) treating the sample under denaturing  
conditions to separate the joined oligonucleotide  
25 product(s) from the target sequence(s), or portion(s)  
thereof, if the target sequence(s), or portion(s)  
thereof, are present in the sample;

-78-

(d) treating the sample with at least two  
oligonucleotide complement pairs, wherein each  
30 oligonucleotide complement pair comprises two  
oligonucleotides selected so as to be sufficiently  
complementary to each other, to each strand of the  
target sequence(s), or portion(s) thereof, and to the  
35 joined oligonucleotide product(s) to hybridize  
therewith and wherein each oligonucleotide is attached  
to a photoactivatable group at the end of the  
oligonucleotide that is adjacent to another  
oligonucleotide when both of the oligonucleotides are  
40 hybridized to a target sequence(s), or portion(s)  
thereof; and

(e) joining the oligonucleotides, hybridized  
to the strands of the target sequence(s), or portion(s)  
thereof, and hybridized to the joined oligonucleotide  
product(s) formed in step (b), to each other by  
45 activating the photoactivatable groups to form a bond  
with adjacent photoactivatable groups, thereby forming  
additional joined oligonucleotide product(s), resulting  
in the amplification of the target sequence(s), or  
portion(s) thereof.

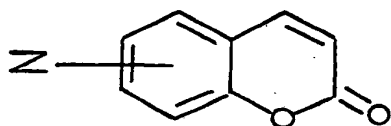
60. The process of claim 59, further  
including step (e') treating the hybridized  
oligonucleotides of step (e) under denaturing  
conditions to separate the hybridized oligonucleotides  
and to produce single-stranded molecules, wherein steps  
5 (d), (e) and (e') are repeated a desired number of  
times.



-79-

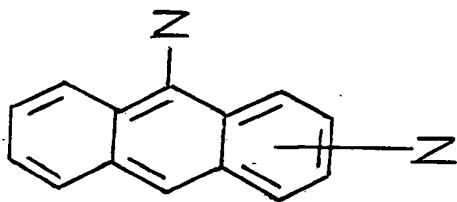
61. The process of claim 59, wherein the oligonucleotide complement pairs are present as a molar excess in the range of  $10^9$  to  $10^{15}$  pairs per nucleic acid target sequence(s), or portion(s) thereof.

62. The process of claim 59, wherein the photoactivatable groups are selected from the group consisting of psoralens,



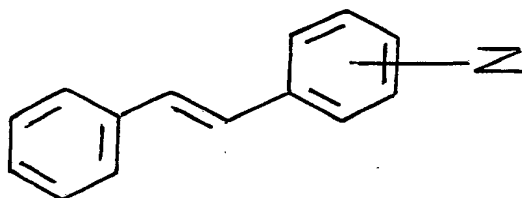
Coumarin derivatives

where Z is H, methoxy, acetoxy,  $C_1-C_5$  alkyl, halogen and di- or tri- derivatives of these groups;



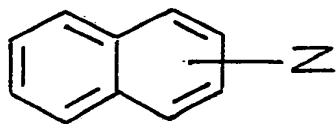
Anthracene derivatives

where Z is H, CN, alkyl or halogen;



Stilbene derivatives

where Z is H, CN, alkyl, halogen,  $CO_2H$ , or alkyl ester;

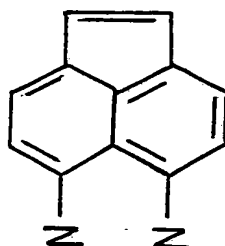


Naphthalene derivatives

-80-

where Z is H, CN, -O-Alkyl, halogen or carboxy; and

derivatives



Acenaphthylene

where Z is H, CN, alkyl, halogen or carboxy.

63. A kit for determining the presence or absence of at least one specific nucleic acid target sequence, or portion(s) thereof, in a sample containing a nucleic acid or a mixture of nucleic acids, or distinguishing between at least two different nucleic acid sequences in the sample, wherein the sample is suspected of containing the target sequence(s), comprising:

at least one container containing at least two oligonucleotide complement pairs, wherein a oligonucleotide complement pair comprises two oligonucleotides selected so as to be sufficiently complementary to each other and to each strand of the target sequence(s), or portion(s) thereof, to hybridize therewith;

a means for mixing the sample with the oligonucleotide complement pairs under hybridizing conditions;

a means for joining an oligonucleotide from a first oligonucleotide complement pair to an oligonucleotide from a second oligonucleotide complement pair, when both oligonucleotides are hybridized to the same strand of a nucleic acid, to

-81-

25 form a joined oligonucleotide product, after the sample  
and oligonucleotide complement pairs are mixed;  
and a means for detecting the joined  
oligonucleotide products.

5 64. The kit of claim 63, further comprising  
a container containing a positive control for  
the oligonucleotide complement pairs which contains the  
specific nucleic acid target sequence(s), or portion(s)  
thereof; and

a container containing a negative control for  
the oligonucleotide complement pairs which does not  
contain the specific nucleic acid target sequence(s),  
or portion(s) thereof.

65. The kit of claim 63, wherein the  
oligonucleotides are oligodeoxyribonucleotides.

5 66. The kit of claim 65, wherein the means  
for joining the oligonucleotide from the first  
oligonucleotide complement pair to the oligonucleotide  
from the second complement pair comprise a DNA  
polymerase, a DNA ligase, and deoxyribonucleotide  
triphosphates.

5 67. The kit of claim 66, wherein the DNA  
polymerase is selected from the group consisting of  
E. coli DNA polymerase-I, Klenow fragments of E. coli  
DNA polymerase-I, T4 DNA polymerase, reverse  
transcriptase and the ligase is selected from the group  
consisting of E. coli DNA ligase or T4 DNA ligase.

-82-

68. The kit claim 66, wherein the DNA polymerase and DNA ligase are heat stable.

69. The kit of claim 66, wherein the DNA polymerase and DNA ligase are immobilized on polymeric supports.

70. The kit of claim 66, wherein the deoxyribonucleotide triphosphates are labelled.

71. The kit of claim 70, wherein the means to detect the joined oligonucleotide product detects the labelled deoxyribonucleotide triphosphates.

5 72. The kit of claim 63, wherein each oligonucleotide is attached to a photoactivatable group at the end of the oligonucleotide that is adjacent to another photoactivatable group at the end of another oligonucleotide, when the oligonucleotides are hybridized to a target sequence(s), or portion(s) thereof.

5 73. The kit of claim 72, wherein the means for joining the oligonucleotide from the first oligonucleotide complement pair to the oligonucleotide from the second complement pair comprises an irradiation source that can excite  $\pi \rightarrow \pi^*$ ,  $n \rightarrow \pi^*$  in the range of 200 nm to 500 nm wavelength.

74. The kit of claim 73, wherein the irradiation source can excite  $\pi \rightarrow \pi^*$ ,  $n \rightarrow \pi^*$  in the range of 300 nm to 400 nm wavelength.

-83-

75. The kit of claim 73, wherein the irradiation source is near U.V. light or ultraviolet laser pulses.

1/3

The Gap

{  
 5--TCCTACAGGGAAGGCCAGGGAATTTCCTTCAGAGCAGACCAGAGCC -3'  
 GGATGTTCCCTTCCGGTCCCTTAAAGAACTCTCCTCTGGTCTCGGT  
 }

The Gap

FIG. 1.

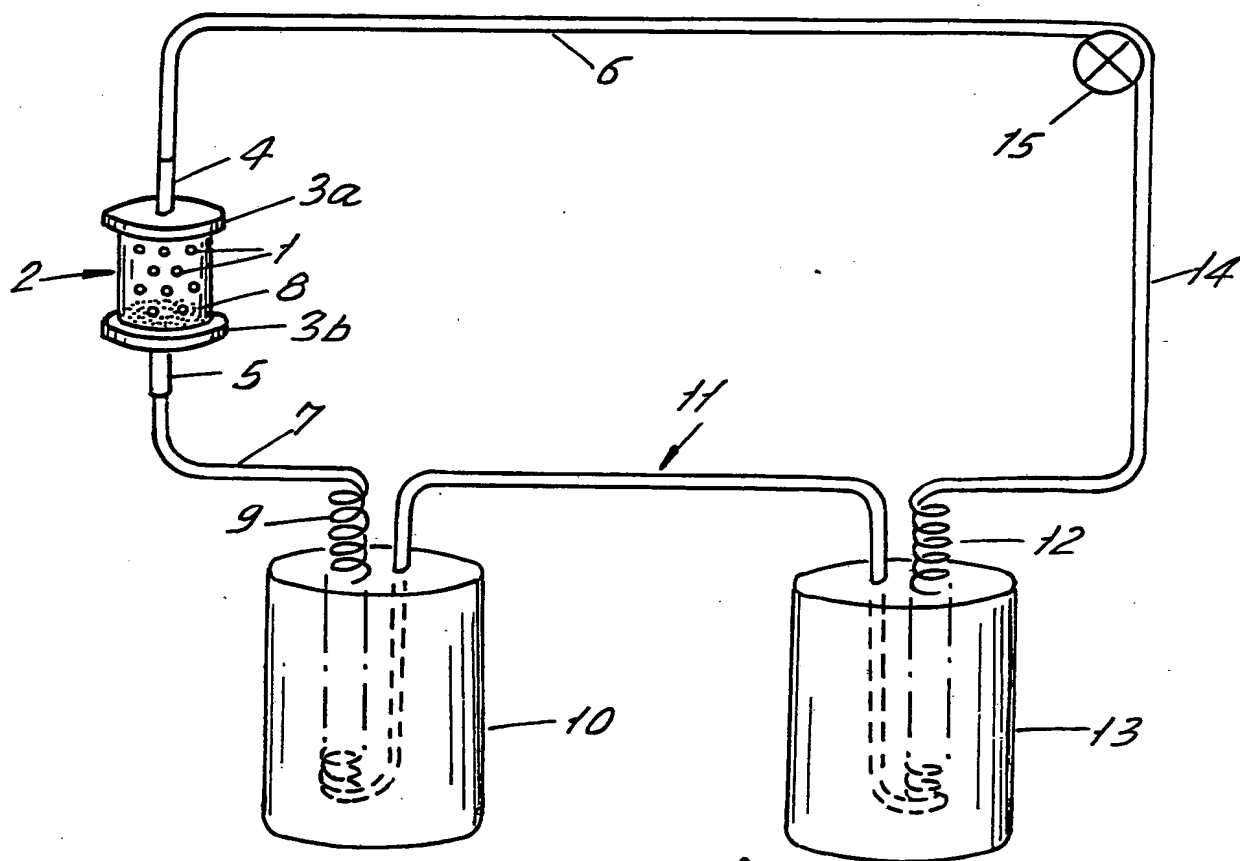
2/3

1 2 3 4 5 6 7 8



FIG. 2.

3/3

FIG. 3.



# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/03125

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC IPC(4): C12Q 1/68; C07H 15/12; G01N 33/48 U.S.Cl.: 435/6; 536/27																	
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Minimum Documentation Searched <sup>7</sup></div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 30%; border-bottom: 1px solid black;">Classification System <sup>1</sup></th> <th style="width: 70%; border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="height: 40px; vertical-align: bottom; border-right: 1px solid black; padding: 5px;">U.S.Cl.</td> <td style="padding: 5px;">435/6; 536/27</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; margin-top: 5px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup></div>			Classification System <sup>1</sup>	Classification Symbols	U.S.Cl.	435/6; 536/27											
Classification System <sup>1</sup>	Classification Symbols																
U.S.Cl.	435/6; 536/27																
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border-bottom: 1px solid black;">Category <sup>*</sup></th> <th style="width: 70%; border-bottom: 1px solid black;">Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup></th> <th style="width: 20%; border-bottom: 1px solid black;">Relevant to Claim No. <sup>13</sup></th> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">US, A, 4,683, 195 (Mullis et al.) 28 July 1987, see abstract.</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-75</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">EP, A, 0,185,494 (Applied Biosystems, Inc.) 25 June 1986, see abstract.</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-27, 32-58 63-71</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">US, A, 4,710,465 (Weissman et al.) 01 December 1987, see column 13 lines, 60-68 and column 15, lines 51-53.</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-27 32-58 63-71</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">DNA, volume 7 number 3, issued April 1988, pages 211-217, NEW YORK; Chang et al.: "Site-Specific Oligonucleotide-Directed Mutagenesis Using T4 DNA Polymerase" see abstract.</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-27 32-58 63-71</td> </tr> </table>			Category <sup>*</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	A	US, A, 4,683, 195 (Mullis et al.) 28 July 1987, see abstract.	1-75	Y	EP, A, 0,185,494 (Applied Biosystems, Inc.) 25 June 1986, see abstract.	1-27, 32-58 63-71	Y	US, A, 4,710,465 (Weissman et al.) 01 December 1987, see column 13 lines, 60-68 and column 15, lines 51-53.	1-27 32-58 63-71	Y	DNA, volume 7 number 3, issued April 1988, pages 211-217, NEW YORK; Chang et al.: "Site-Specific Oligonucleotide-Directed Mutagenesis Using T4 DNA Polymerase" see abstract.	1-27 32-58 63-71
Category <sup>*</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>															
A	US, A, 4,683, 195 (Mullis et al.) 28 July 1987, see abstract.	1-75															
Y	EP, A, 0,185,494 (Applied Biosystems, Inc.) 25 June 1986, see abstract.	1-27, 32-58 63-71															
Y	US, A, 4,710,465 (Weissman et al.) 01 December 1987, see column 13 lines, 60-68 and column 15, lines 51-53.	1-27 32-58 63-71															
Y	DNA, volume 7 number 3, issued April 1988, pages 211-217, NEW YORK; Chang et al.: "Site-Specific Oligonucleotide-Directed Mutagenesis Using T4 DNA Polymerase" see abstract.	1-27 32-58 63-71															
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>*</sup> Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p> </div> </div>																	
<b>IV. CERTIFICATION</b> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">Date of the Actual Completion of the International Search</td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">Date of Mailing of this International Search Report</td> </tr> <tr> <td style="padding: 5px;">30 October 1989</td> <td style="text-align: center; padding: 5px;"><b>21 NOV 1989</b></td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;">International Searching Authority</td> <td style="border-bottom: 1px solid black; padding: 5px;">Signature of Authorized Officer</td> </tr> <tr> <td style="padding: 5px;">ISA/US</td> <td style="text-align: center; padding: 5px;">             SCOTT A. CHAMBERS         </td> </tr> </table>			Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	30 October 1989	<b>21 NOV 1989</b>	International Searching Authority	Signature of Authorized Officer	ISA/US	 SCOTT A. CHAMBERS							
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report																
30 October 1989	<b>21 NOV 1989</b>																
International Searching Authority	Signature of Authorized Officer																
ISA/US	 SCOTT A. CHAMBERS																

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☒ **FADED TEXT OR DRAWING**
- ☒ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**